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67. THE NUTRITIVE VALUE OF PROTEINS FOR MILK PRODUCTION

II. A COMPARISON OF THE PROTEINS OF BLOOD MEAL, PEA MEAL, DECORTICATED EARTH-NUT CAKE, AND A MIXTURE OF DECORTICATED EARTH-NUT CAKE AND FLAKED MAIZE

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(With 4 Figures.)

IN a previous paper(1) it was shown that the deficiency of an essential amino acid, such as lysine or tryptophane, from the rations of milking cows, resulted in a relatively poor utilisation of protein for milk production. This conclusion was based on experiments in which the main sources of protein were bean meal, linseed-oil meal, linseed cake, and meat meal. In the present paper the results of a similar study are presented, the sources of protein being blood meal, pea meal, decorticated earth-nut cake, and flaked maize. It will be seen from Table I that the lysine content of each of the two former foods is relatively high, while the two latter foods are markedly deficient in this amino acid.

Table I*. *Amino-acid content of foods* (*as percentage of total* N).

	Lysine	Tryptophane	Cystine	Histidine *plus* arginine
Separated milk	8·34	4·54	2·04	15·32
Blood meal	11·04	6·51	2·65	17·91
Pea meal	7·04	2·56	1·55	14·14
Decorticated earth-nut cake	4·46	5·20	1·00	25·11
Flaked maize	2·15	1·78	1·48	10·65

* The figures in this table were obtained by a modification of Van Slyke's nitrogen distribution method. The tryptophane figures represent insoluble humin, and can only be taken as a rough estimate of the tryptophane content.

EXPERIMENTAL METHODS.

(*a*) *Animals.*

Three pedigree Ayrshire cows were used, each animal being in her third lactation and from 4 to 6 weeks calved. The weights of the cows varied from 950 to 1000 lb.

(*b*) *Rations.*

The original plan was to run the experiment in five feeding periods, in order that the ration for the final period might be identical with that for the first. One of the cows, Dunlop, started the experiment with a rather low milk yield,

i.e. about 30 lb. daily, and it was found impracticable to carry her beyond the second feeding period. The two remaining animals, Treeshill and Knockbirnie, completed the five periods.

In order that reliable data should be available for the calculation of biological values (see p. 10) each feeding period was continued until a fairly steady milk yield had been maintained for at least 14 days. Consequently the length of the complete feeding periods varied. Thus, in one instance the

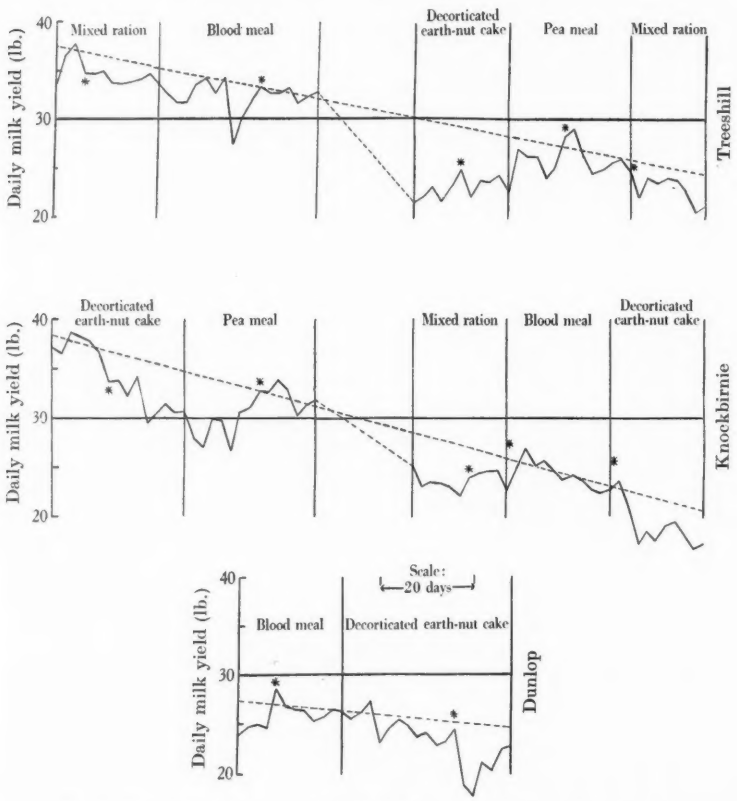

Fig. 1. Showing variations in milk yield during successive feeding periods. (*Note.* Asterisk indicates period used in calculation of biological values.)

complete period extended over 34 days, while in another it only lasted for 16 days. With the exception of Tables X–XII, the data in this paper have been calculated for the *complete* feeding periods.

Throughout the experiment each cow received a maintenance ration of straw, oats and beet pulp, at the rate of 6 lb. starch equivalent and 0·6 lb. protein equivalent per 1000 lb. live weight. The production rations were fed at the rate of 2·5 lb. starch equivalent and 0·44 lb. protein equivalent per

10 lb. milk, as in the previous work. The concentrates[1] under investigation were fed in the production ration in quantities which supplied equivalent amounts of nitrogen to the ration, the rations being balanced with oats and starch, as before. Water was allowed *ad lib.*, but was measured by meter.

At the completion of the second period the animals had to be removed from the metabolism stalls for 21 days to enable repairs and cleaning to be carried out. During this period a deficient ration was fed, in order to avoid the possibility of protein storage. This accounts for the marked fall in milk yields shown in Fig. 1.

The actual rations fed are shown in Table II, while in Table III a comparison is given of the lysine content of each ration.

Table II. *Details of daily rations.*

Treeshill		Knockbirnie	
Maintenance		Maintenance	
	4·550 kg. straw		4·550 kg. straw
	1·500 kg. beet pulp		1·500 kg. beet pulp
	1·700 kg. oats		1·700 kg. oats
Production		Production	
Period 1:	1·700 kg. oats	Period 1:	5·100 kg. oats
	1·275 kg. decorticated earth-nut cake		0·773 kg. decorticated earth-nut cake
	2·125 kg. flaked maize		1·540 kg. starch
	1·510 kg. starch	Period 2:	5·100 kg. oats
Period 2:	5·100 kg. oats		1·591 kg. pea meal
	0·424 kg. blood meal		1·510 kg. starch
	1·773 kg. starch	Period 3:	1·700 kg. oats
Period 3:	5·100 kg. oats		1·275 kg. decorticated earth-nut cake
	0·773 kg. decorticated earth-nut cake		2·125 kg. flaked maize
	1·540 kg. starch		1·510 kg. starch
Period 4:	5·100 kg. oats	Period 4:	5·100 kg. oats
	1·591 kg. pea meal		0·424 kg. blood meal
	1·510 kg. starch		1·773 kg. starch
Period 5:	Same as (1)	Period 5:	Same as (1)

Dunlop	
Maintenance	
	4·550 kg. straw
	1·500 kg. beet pulp
	1·700 kg. oats
Production	
Period 1:	5·100 kg. oats
	0·340 kg. blood meal
	0·416 kg. starch
Period 2:	5·100 kg. oats
	0·549 kg. decorticated earth-nut cake
	0·250 kg. starch

Table III. *Lysine content of rations (as percentage of total N fed per day).*

Period	Treeshill	Knockbirnie	Dunlop
1	3·36	3·49	5·31
2	5·70	4·31	3·07
3	3·49	3·36	—
4	4·31	5·70	—
5	3·36	3·49	—

[1] The blood meal used in the investigation was prepared from sheep's blood by a special low-temperature process employed by Messrs W. and J. Dunlop, Ltd., of Dumfries. The remaining concentrates were purchased on the open market.

Discussion of results.

(*a*) *Milk yield.*

In the previous paper it was shown that the feeding of a deficient protein, at the low level of 0·44 lb. protein equivalent per 10 lb. milk, ultimately led to a reduction in milk yield. Reference to Fig. 1 shows that this conclusion has been confirmed in the present investigation. In this figure the dotted line shows the expected lactation curve for an animal fed throughout the experiment on an adequate protein (blood meal or pea meal). It will be seen that feeding with decorticated earth-nut cake, or with a mixed ration low in lysine (*i.e.* decorticated earth-nut cake and flaked maize), causes a definite fall in milk yield, while a change to a ration containing a good quality protein results in a return to the normal milk yield. Moreover, the relatively prolonged period between the change in ration and the fall from, or return to, the normal lactation curve again indicates in the one case the utilisation, and in the other the formation, of reserve N in the body. Further evidence of this utilisation of reserve N will be given later.

(*b*) *Total nitrogen of milk and urine.*

Figs. 2, 3 and 4 show the relationship between the total output of N in the milk and the urine during the five experimental periods. In the previous paper

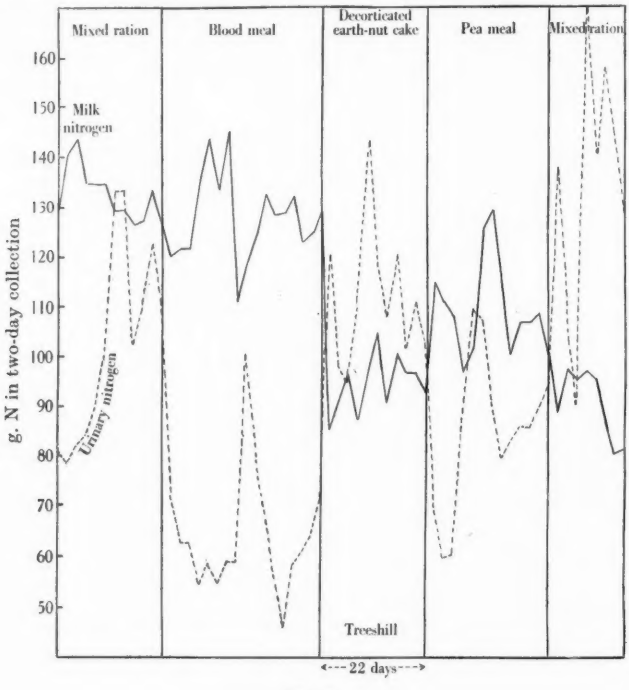

Fig. 2.

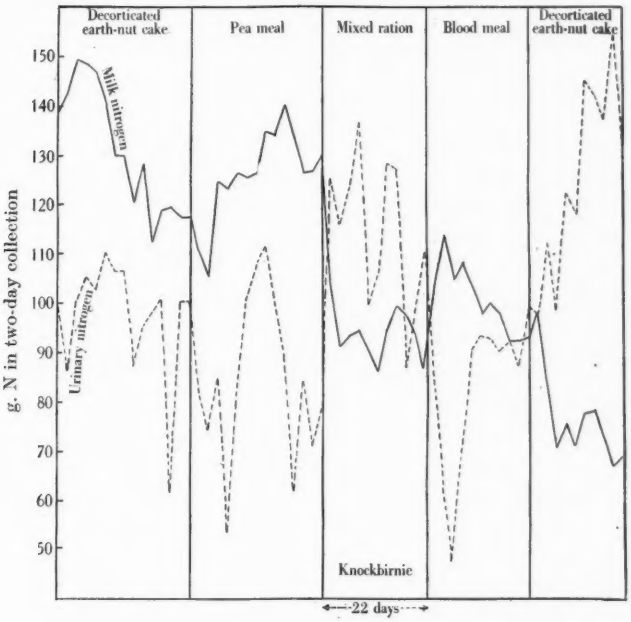
Decorticated earth-nut cake
Pea meal
Mixed ration
Blood meal
Decorticated earth-nut cake
Milk nitrogen
Urinary nitrogen
g. N in two-day collection
150
140
130
120
110
100
90
80
70
60
50
Knockbirnie
22 days

Fig. 3.

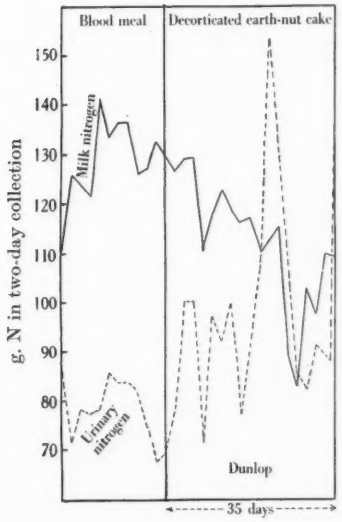
Blood meal
Decorticated earth-nut cake
Milk nitrogen
Urinary nitrogen
g. N in two-day collection
150
140
130
120
110
100
90
80
70
Dunlop
35 days

Fig. 4.

results were only given for the last 14 days of each feeding period, whereas the present figures relate to each complete feeding period. In interpreting the results allowance must be made for the gradual increase in urinary N resulting from the normal decrease in milk yield during the lactation period, since the quantity of protein fed throughout the experiment was constant, being based on the initial milk yield.

The results show clearly that a relatively high nitrogen wastage accompanies the feeding of the deficient proteins, whereas the wastage is markedly lower on the rations containing the lysine-rich proteins.

(*c*) *Nitrogen partition in the urine.*

Figures for the total urinary N and the N partition products averaged for each complete feeding period are shown in Tables IV and V.

Table IV. *Urinary excretion of nitrogen* (*average 2-day output for each complete feeding period*).

Cow	Period	Urea N g.	Ammonia N g.	Amino-acid N g.	Creatinine g.	Creatine g.	Total N g.
Treeshill	Mixed ration	60·23	16·19	2·68	9·71	7·11	102·55
	Blood meal	34·48	11·51	1·74	9·10	3·14	64·64
	Decorticated earth-nut cake	56·12	28·59	3·08	10·74	5·97	111·61
	Pea meal	52·73	12·50	2·91	10·60	3·21	83·95
	Mixed ration	72·11	32·98	4·13	10·20	8·41	134·41
Knockbirnie	Decorticated earth-nut cake	57·94	19·23	1·94	9·78	5·69	97·51
	Pea meal	50·15	15·34	2·35	9·56	3·55	87·63
	Mixed ration	63·19	26·08	2·49	10·49	5·82	114·32
	Blood meal	54·84	11·76	2·29	10·70	3·17	83·16
	Decorticated earth-nut cake	68·56	30·01	4·72	10·56	6·93	126·07
Dunlop	Blood meal	51·32	9·95	1·60	9·45	4·87	78·35
	Decorticated earth-nut cake	50·06	27·28	3·71	8·83	8·61	99·84

Table V. *Urinary partition of nitrogen* (*as percentage of total urinary nitrogen*).

Cow	Period	Urea N	Ammonia N	Amino-acid N
Treeshill	Mixed ration	58·7	15·8	2·6
	Blood meal	53·4	17·8	2·7
	Decorticated earth-nut cake	50·3	25·6	2·8
	Pea meal	62·8	14·9	3·4
	Mixed ration	53·7	24·6	3·0
Knockbirnie	Decorticated earth-nut cake	59·3	19·5	2·0
	Pea meal	57·2	17·5	2·7
	Mixed ration	55·3	22·8	2·2
	Blood meal	66·0	14·2	2·8
	Decorticated earth-nut cake	54·4	23·8	3·8
Dunlop	Blood meal	65·5	12·8	2·0
	Decorticated earth-nut cake	50·1	27·3	3·7

It will be seen from Table IV that the feeding of the deficient proteins results in a marked increase in the excretion of exogenous metabolites through the deamination of the excess circulating amino acids. As regards N partition,

the results are not sufficiently consistent to warrant any generalisation, though there is some indication that the percentage of ammonia N is higher on the deficient protein rations—a result which was also obtained with the linseed rations in the previous work. There are also no significant variations in the output of amino-acid N during the five periods.

With regard to endogenous metabolism, the variations in the output of creatine are striking. It will be seen from Table IV that the creatine excretion increased by over 100 per cent. during the periods in which the lysine-deficient rations were fed—a clear demonstration of the utilisation of tissue N by the animals in their attempts to maintain milk production on the poor quality proteins.

(*d*) *Sulphur excretion in the urine.*

The recent work of Wilson[2, 3, 4, 5] has re-directed attention to the important rôle of sulphur in the study of protein metabolism. In the present investigation estimations were made (by Denis's method) of the sulphur content of the food and urine during each feeding period. The results are shown in Table VI, in which the S : N ratios of both the food and the urine are also recorded.

Table VI. *Nitrogen and sulphur output in urine (average 2-day output for each complete feeding period).*

Cow	Ration	Urinary N g.	Urinary S g.	S : N ratio	S : N ratio of ration
Treeshill	Mixed ration	109·87	9·65	1 : 11·4	1 : 13·9
	Blood meal	61·50	8·95	6·9	10·9
	Decorticated earth-nut cake	111·60	9·60	11·6	13·9
	Pea meal	83·95	10·35	8·1	12·0
	Mixed ration	134·41	10·90	12·3	13·9
Knockbirnie	Decorticated earth-nut cake	95·25	10·43	9·1	13·9
	Pea meal	85·26	11·38	7·5	12·0
	Mixed ration	114·30	11·30	10·1	13·9
	Blood meal	83·16	10·98	7·6	10·9
	Decorticated earth-nut cake	126·07	12·05	10·5	13·9
Dunlop	Blood meal	78·35	9·69	8·1	10·9
	Decorticated earth-nut cake	99·84	9·00	11·1	13·9

It will be noted that the S : N ratio of the urine is in every case greater than that of the ration fed. This would be anticipated since, as the S : N ratio of milk is only 1 : 18, there is a *relative* excess of sulphur in the ingested food. This excess will be eliminated in the urine, with a consequent rise in the S : N ratio. For example, the pea-meal ration has a S : N ratio of 1 : 12. The ingestion of 96 g. N from this ration will therefore be accompanied by an equivalent ingestion of 8 g. S. If 54 g. N are secreted in the milk, there will be an equivalent secretion of only 3 g. S. On the assumption that the ingested ration is completely assimilated and that no nitrogen or sulphur are required for wear and tear of body tissue, the quantities excreted in the urine will be 42 g. N and 5 g. S, giving a S : N ratio of 8·4.

Attention has already been drawn to the fact that during the poor quality protein periods there is a marked utilisation of body tissue. Since the S : N

ratio of body tissue is constant (*circa* 15·7), it should be possible, by calculating the S : N ratios of the excess N and excess S excreted during the poor protein periods, to secure additional evidence on this point.

Since the nitrogen intake for all periods is approximately constant, the excess N on the deficient protein rations can be arrived at by subtracting the urinary N on an adequate ration from that on the deficient ration. It is, however, impossible to use a similar calculation in estimating the excess S, since the intake of sulphur varied in the different periods. An attempt has therefore been made to apply a correction to the observed values which will render the figures for sulphur excretion comparable for each period. Thus, using the data for Treeshill's mixed ration and blood-meal periods, it will be observed that the quantities of sulphur ingested were 14·64 g. and 18·10 g. respectively. On the assumption that, had this extra 3·45 g. S been ingested in the mixed ration period a proportionate quantity of sulphur would have been assimilated and excreted in the urine, the urinary S for the mixed ration has been arbitrarily multiplied by 18·10/14·64, or 1·25. This assumption is not unreasonable, since it has already been shown that, on a lysine-deficient ration, the extra sulphur intake could not have been used for milk production.

The results of the above calculations, together with the ratios of excess S to excess N, are shown in Table VII. It should be noted that, in calculating

Table VII. *Excess nitrogen and sulphur excreted in urine (for each complete feeding period).*

Cow	Ration	Excess N g.	Excess S g.	S : N ratio	S : N ratio of ration
Treeshill	Mixed ration	48·37	3·11	1 : 15·6	1 : 13·9
	Blood meal	—	—	—	10·9
	Decorticated earth-nut cake	50·10	3·20	15·7	13·9
	Pea meal	22·45	1·40	16·0	12·0
	Mixed ration	72·91	4·66	15·7	13·9
Knockbirnie	Decorticated earth-nut cake	10·00	0·63	16·0	13·9
	Pea meal	—	—	—	12·0
	Mixed ration	29·00	1·70	17·1	13·9
	Blood meal	—	—	—	10·9
	Decorticated earth-nut cake	40·81	2·60	15·7	13·9
Dunlop	Blood meal	—	—	—	10·9
	Decorticated earth-nut cake	21·50	1·64	13·4	13·9

the excess values, the figures for the S and N excretion on the adequate rations have been based on the second feeding periods, in order to avoid complications due to the natural fall in milk yield, and the consequent decreased utilisation of ingested N and S for milk production, in the fourth periods. Thus the blood-meal period is used as a basis of comparison with Treeshill, while the pea-meal period is used with Knockbirnie. It will be seen from Table VII that the S : N ratios of the excess sulphur and nitrogen excreted vary closely around the ratio for body tissue, *i.e.* 1 : 15·7. The results therefore confirm the conclusions already reached, namely that when poor quality protein rations are fed, the lactating animal catabolises its own body tissue in an attempt to make good the deficiency in essential amino acids required for milk production.

(*e*) *Total output of milk, urine and faeces.*

In Table VIII the intake of food and water are compared with the output of milk, urine and faeces.

Table VIII. *Comparison of total intake with total output (2-day average for each complete feeding period).*

		Intake		Output		
Cow	Feed	Quantity kg.	Water gal.	Urine c.c.	Faeces kg.	Milk lb.
Treeshill	Mixed ration	14·36	13·6	6542	40·53	69·73
	Blood meal	15·05	13·7	7057	54·47	64·92
	Decorticated earth-nut cake	15·16	13·0	6268	57·36	46·23
	Pea meal	15·95	13·0	7206	47·79	52·15
	Mixed ration	14·36	11·0	7606	42·74	45·60
Knockbirnie	Decorticated earth-nut cake	15·16	13·0	6981	30·17	68·32
	Pea meal	15·95	12·8	9393	39·27	61·55
	Mixed ration	14·36	12·6	8752	34·83	47·36
	Blood meal	15·05	13·0	7045	30·50	48·59
	Decorticated earth-nut cake	15·16	10·8	7206	34·46	37·88
Dunlop	Blood meal	13·61	12·2	6721	37·92	51·85
	Decorticated earth-nut cake	13·65	13·0	8327	46·53	46·57

(*f*) *Faecal nitrogen.*

In the previous paper(1) it was shown that the faecal N on the deficient protein ration (linseed) was significantly lower than that on the adequate protein ration (bean meal). With one exception (Knockbirnie, period 2) the results in the present paper (Table IX) confirm the previous observations. The

Table IX. *Variation in nitrogen output of faeces (average 2-day output for each complete feeding period).*

Cow	Feed	Faecal N (g.)
Treeshill	Mixed ration	172·35
	Blood meal	186·40
	Decorticated earth-nut cake	148·33
	Pea meal	193·95
	Mixed ration	161·27
Knockbirnie	Decorticated earth-nut cake	159·40
	Pea meal	168·98
	Mixed ration	178·52
	Blood meal	182·40
	Decorticated earth-nut cake	160·09
Dunlop	Blood meal	166·20
	Decorticated earth-nut cake	155·39

variations in faecal N excretion are not due to normal differences in digestibility, the values for the digestibility of the composite production rations, calculated from Wood's tables, being reasonably constant for all periods.

	Blood meal	Pea meal	Mixed ration	Decorticated earth-nut cake
Percentage of crude protein digestible (Wood's tables)	90	86	93	90

It therefore seems reasonable to assume that, where minimal quantities of protein are fed, the absorption of N from, or the re-excretion of N into, the gut is largely dependent on the quality of the protein ingested.

(*g*) *Nitrogen balance.*

Special attention was drawn in the previous paper to the positive nitrogen balances found during the 14-day experimental periods. In the present series analyses were made throughout the complete feeding periods, including the transitional periods, so that nitrogen balances could be calculated for both the complete and the experimental periods[1] (Table X). It will be seen that there is no consistency in the values for the various periods, nor can the balances for the complete periods be correlated with those for the experimental periods. There is some indication of a slightly greater N retention in the complete periods than in the experimental periods, but the differences are not significant. The results demonstrate the danger of basing accurate nitrogen metabolism studies on short period experiments.

(*h*) *Calculation of biological values.*

The data used for calculating the biological values of the various rations are given in Table XI. The formula used was that noted in the previous paper (1), *i.e.*

$$\text{Biological value} = \frac{X}{I_{(\text{corr.})} - (F_t - M_t) + (F_m - M_m) + (F_x - M_x)}.$$

As in the previous work, the figure for metabolic faecal N has been based on that found for a steer, *i.e.* 0·405 g. N per 100 g. dry matter intake. The following figures, abstracted from Table XI, show the close agreement between the biological values obtained with the three animals:

Ration	Treeshill	Knockbirnie	Dunlop	Mean, omitting Dunlop
Blood meal	72·5	74·3	65·1	73·4
Pea meal	69·0	60·2	—	64·6
Mixed ration	53·5 and 50·9	51·6	—	52·0
Decorticated earth-nut cake	48·3	48·7 and 52·9	51·6	49·9

It must again be emphasised that these figures refer, not to the pure foods, but to these foods supplemented by oats. Account must also be taken of the relatively high maintenance intake, and to the possible supplementary relationship between the proteins of the maintenance ration and of the production ration. With these reservations, it may be concluded that blood meal and pea meal provide a better source of nitrogen for milk production than decorticated earth-nut cake or decorticated earth-nut cake *plus* flaked maize, the relative values of the rations containing these concentrates being: blood meal 73, pea meal 65, decorticated earth-nut cake *plus* flaked maize 52, and decorticated earth-nut cake 50.

(*k*) *Correlation of biological values with amino-acid composition.*

It has been assumed throughout both this and the previous paper that any observed differences in the utilisation of the various rations were due to differences in the amino-acid content of the concentrates. If this assumption is true, it might be expected that a direct correlation would exist between the

[1] The commencement of each experimental period is marked with an asterisk in Fig. 1.

Table X. *Nitrogen balances for each complete feeding period.*

Cow	Period	Intake g. N	Output g. N	Gain or loss for period g. N	Daily gain or loss g. N	Daily gain or loss (calculated for experimental periods only) g. N
Treeshill	Mixed ration	4886·00	4888·68	− 2·68	−0·11 (24 days)	− 4·94 (18 days)
	Blood meal	6448·50	6441·74	+ 6·76	+0·20 (34 ,,)	+11·57 (14 ,,)
	Decorticated earth-nut cake	4079·15	3897·21	+181·94	+8·30 (22 ,,)	− 4·10 (12 ,,)
	Pea meal	5200·00	5028·44	+171·56	+6·60 (26 ,,)	+ 1·30 (16 ,,)
	Mixed ration	3084·40	3085·37	− 0·97	— (16 ,,)	— (16 ,,)
Knockbirnie	Decorticated earth-nut cake	5844·00	5812·27	+ 31·73	+1·06 (30 ,,)	+ 1·01 (18 ,,)
	Pea meal	5400·00	5357·67	+ 42·33	+1·50 (28 ,,)	− 1·80 (14 ,,)
	Mixed ration	4271·70	4252·02	+ 19·68	+0·90 (22 ,,)	— (12 ,,)
	Blood meal	4180·00	4028·31	+151·69	+6·90 (22 ,,)	+ 6·90 (22 ,,)
	Decorticated earth-nut cake	3800·00	3637·94	+162·06	+8·10 (20 ,,)	+ 8·10 (20 ,,)
Dunlop	Blood meal	4560·00	4480·11	+ 79·89	+3·33 (24 ,,)	− 1·79 (16 ,,)
	Decorticated earth-nut cake	6185·44	6249·01	− 63·57	−1·87 (34 ,,)	+ 6·14 (14 ,,)

Table XI. *Biological values.*

Cow	Period	% decrease in milk N from first period	X	$I_{(corr.)}$	F_t	M_t	F_m	M_m	F_x	M_x	Biological value
Treeshill	Mixed ration	—	65·35	147·64	92·40	57·44	39·34	29·84	—	—	53·5
	Blood meal	8·2	64·23	119·48	101·20	60·20	39·34	29·84	2·51	1·86	72·5
	Decorticated earth-nut cake	20·4	48·51	108·41	78·89	60·64	39·34	29·84	5·16	4·29	48·3
	Pea meal	25·9	55·29	105·37	99·28	63·80	39·34	29·84	7·32	6·61	69·0
	Mixed ration	31·3	45·00	101·43	80·63	57·44	39·34	29·84	8·05	7·32	50·9
Knockbirnie	Decorticated earth-nut cake	—	60·75	136·19	81·51	60·64	39·34	29·84	—	—	48·7
	Pea meal	9·5	66·23	128·86	92·93	63·80	39·34	29·84	2·60	1·91	60·2
	Mixed ration	25·2	47·14	110·73	89·36	59·44	39·34	29·84	5·01	4·00	51·6
	Blood meal	32·0	50·33	88·50	91·20	60·20	39·34	29·84	8·01	7·28	74·3
	Decorticated earth-nut cake	40·1	38·41	81·58	80·05	60·64	39·34	29·84	9·14	8·19	52·9
Dunlop	Blood meal	—	66·50	121·23	83·10	54·44	39·34	29·84	—	—	65·1
	Decorticated earth-nut cake	5·6	50·80	114·60	80·48	54·60	39·34	29·84	1·63	1·30	51·6

Where X = total milk N; $I_{(corr.)}$ = production N intake corrected for milk yield; F_t = total faecal N; F_m = maintenance faecal N; F_x = faecal N corrected for production intake; M_t = total metabolic N; M_m = maintenance metabolic N; M_x = metabolic N corrected for production intake.

biological value of the ration and the percentage of the amino acid which constitutes the limiting factor in the ration (*i.e.* lysine or tryptophane). Table XII shows a comparison of these values for both the present work and the previous series of experiments. It will be seen that there is in fact a very close correlation between the relative lysine (or tryptophane) contents of the rations and their biological values.

Table XII. *Correlation between biological value and amino-acid content.*

Cow	Ration	Lysine supplied g.	Trypto-phane supplied g.	% difference	Bio-logical value	% difference
Treeshill	Mixed ration	7·01	—	28·9	52	28·8
	Blood meal	*9·86*	—	—	*73*	—
	Decorticated earth-nut cake	6·84	—	30·63	48	34·2
	Pea meal	8·35	—	15·30	69	5·5
Knockbirnie	Decorticated earth-nut cake	6·84	—	30·6	51	31·1
	Pea meal	8·35	—	15·3	60	18·9
	Mixed ration	7·01	—	28·9	52	29·7
	Blood meal	*9·86*	—	—	*74*	—
Dunlop	Blood meal	*8·81*	—	—	*65*	—
	Decorticated earth-nut cake	6·18	—	29·8	52	20·0
Mona	Beans	*8·45*	*10·85*	—	*60*	—
	Linseed	6·03	—	28·0	46	23·3
	Meat meal	—	9·85	9·2	55	8·3
Butterfly	Beans	*8·45*	*10·85*	—	*63*	—
	Linseed	6·03	—	28·0	48	24·0
	Meat meal	—	9·85	9·2	63	Nil
Milkmaid	Beans	*8·10*	—	—	*70*	—
	Linseed	6·18	—	24·0	61	13·0
Kate	Beans	*8·37*	*9·87*	—	*54*	—
	Linseed	6·45	—	23·0	44	18·5
	Meat meal	—	8·87	10·0	48	11·0

Note. The figures in italics were taken as standard values from which the percentage differences were calculated.

It should be noted, however, that the amino-acid contents have in all cases been calculated on the complete ration fed, *i.e.* both the maintenance and production rations. It was found that calculations based on the production ration alone failed to give such close correlations. This fact indicates that the amino acids in the maintenance ration are capable of supplementing those in the production ration, and that only minimal amounts of lysine (and tryptophane) are required for adult maintenance.

The close correlations shown in Table XII do, however, show that a close approximation to the biological value of a food protein may be obtained by a determination of its amino-acid content.

Summary.

The results of the present experiments and of those reported in the previous paper(1) may be summarised as follows:

1. When minimal quantities of protein are fed in the production rations of milking cows, a deficiency of either lysine or tryptophane will lead to a marked reduction in milk yield. There is, however, some evidence of the storage of reserve N, which can be utilised when the food protein is inadequate.

2. The feeding of a lysine- or tryptophane-deficient ration causes a marked increase in urinary N, indicating a poor utilisation of food protein. On the other hand, the feeding of a ration containing adequate quantities of these essential amino acids reduces the urinary N, indicating efficient protein utilisation.

3. The utilisation of body tissue in an attempt to maintain normal milk production on a deficient protein ration is shown by the high creatine excretion. The fact that the S : N ratio of the excess sulphur and nitrogen excreted during the deficient protein periods approximates that of body tissue (*circa* 1 : 15·7) confirms this conclusion.

4. As regards urinary partition of nitrogen, there is some indication that the percentage of urea N decreases and the percentage of ammonia N increases on the deficient protein rations.

5. Evidence is available to show that, where minimal quantities of protein are fed, the absorption of nitrogen from, or the re-excretion of nitrogen into, the gut is largely dependent on the quality of the protein ingested.

6. The marked variation in the nitrogen balances, not only on different rations but during different periods on the same ration, demonstrates that such data cannot be used as a measure of the utilisation of food protein.

7. A new formula has been devised for calculating the relative biological values of different proteins for milk production. By applying this formula to the experiments reported in this and the previous paper, the following biological values are found:

Blood meal	73
Pea meal	64
Bean meal	59
Meat meal	55
Decorticated earth-nut cake *plus* flaked maize ...	52
Decorticated earth-nut cake	50
Linseed-oil meal or linseed cake	46

These values refer, not to the pure foods, but to these supplemented by oats and fed with a maintenance ration of constant composition. There is evidence of a supplementary relationship between the amino acids of the maintenance and production rations.

8. There is a remarkably close correlation between the biological value of the ration and its content of lysine or tryptophane.

37717

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637.128:576.8

68. THE OCCURRENCE OF COLIFORM BACTERIA IN MILK

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(With 1 Figure.)

Bacterial tests are of great value for determining the extent to which samples of milk have been contaminated. While the estimation of the total viable bacterial content is most commonly employed, it suffers from the disadvantage that it gives as a rule little or no indication of the nature and origin of the bacterial contaminants. Consequently for this purpose additional tests are commonly carried out. Of these tests the determination of the prevalence of coliform organisms in the milk, the coliform test, is the most frequently used, as these organisms are recognised to be the most important of all the non-pathogenic bacterial contaminants of milk. Many of the coliform bacteria occur commonly in animal excreta while others occur less frequently in faeces but are fairly common in soil and even on plants and cereal grains. Accordingly their presence in milk is widely accepted as a reliable index of the extent of its contamination with extraneous bacteria.

The purpose of the present work was (*a*) to determine the prevalence of coliform bacteria in market milk at different seasons of the year, (*b*) to correlate their presence with the total bacterial content, and (*c*) to examine the biological reactions of coliform organisms isolated from milk with a view to ascertaining, if possible, their origin from direct faecal contamination or from external sources.

(*a*) The prevalence of coliform bacteria in milk.

The prevalence of coliform bacteria was determined in 21,569 samples of market milk which had been tested by the official coliform test(1). The samples in most instances were taken without warning from the milk supplies of individual farmers as they were being weighed in the receiving tank at a creamery or dairy. They are therefore typical of the general supplies. The tests extended over a period of several years. Of the 21,569 samples, 48·3 per cent. gave negative coliform results with 1/10, 1/100 and 1/1000 c.c.; 21·4 per cent. gave positive results with 1/10 and negative results with 1/100 and 1/1000 c.c.; 14·0 per cent. gave positive results with 1/10 and 1/100 c.c. and negative with 1/1000 c.c.; and 16·3 per cent. gave positive results with 1/10, 1/100 and 1/1000 c.c. It is probable that these results are better than those generally obtained from samples of ordinary farm milk because all were tested

Table I. *The influence of season and temperature on the proportion of coliform-positive samples* and on the bacterial content of samples of milk from the same group of farms.*

Average number of samples per month—30.

Month in which samples received	Bacterial content per c.c.		Proportion of coliform-positive samples, expressed as a percentage	Mean of the minimum and maximum atmospheric temperatures for the day on which samples received
	Mean	Median		
January	21,304	12,400	24·0	31·5
February	33,179	22,000	35·5	41·0
March	29,407	160,000	15·0	42·0
April	33,263	11,650	41·0	42·5
May	46,126	14,800	39·0	48·5
June	112,444	27,200	63·0	55·5
July	277,859	118,400	96·0	57·5
August	252,431	158,500	91·0	57·0
September	90,488	27,800	62·0	55·0
October	26,131	10,900	47·0	45·5
November	9,950	5,300	25·0	47·5
December	11,764	9,800	24·0	39·5

* Samples containing coliform organisms in 1/10 c.c.

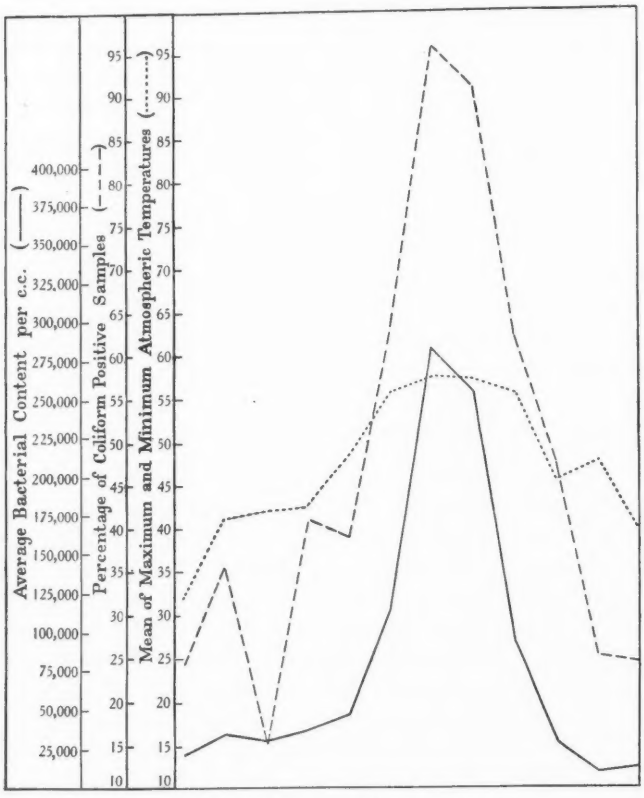

Fig. 1. Graphic representation of Table I.

within 24 hours and many within 12 hours of milking. Further, they were obtained mostly from creameries whose supplies have been submitted to such tests for a number of years.

The influence of season on the prevalence of coliform bacteria in milk.

Coliform organisms are much more prevalent in samples of milk during the warmer months of the year, as is evident when the results of the tests for a whole year from the same group of farms are examined. The tests were made at monthly intervals; all the tests for one month were performed on the same day, and the mean of the minimum and maximum atmospheric temperatures estimated for that day (see Table I and Fig. 1). Although the table gives the results for only one group of farms, it is typical of those for other groups, and shows that there is a definite positive correlation between the proportion of coliform positive samples and the mean of the minimum and maximum atmospheric temperatures. This is probably due largely to the influence of temperature on the rate of multiplication of the bacterial contaminants, both in milk which has not been cooled and in utensils which have not been properly sterilised.

(*b*) THE RELATION OF THE PRESENCE OF COLIFORM BACTERIA TO THE TOTAL BACTERIAL CONTENT.

Samples of milk which do not contain coliform organisms in 1/10 c.c. ("coliform negative") have generally a lower total bacterial content than those which contain them in 1/10 c.c. ("coliform positive"). In an analysis of the bacterial tests on 21,857 samples of milk, estimations were made of the average bacterial content per c.c. of the coliform-negative and coliform-positive samples; 10,458 samples were coliform negative and had an average bacterial content of 25,294 per c.c., whereas 11,399 samples which were coliform positive had an average bacterial content of 160,577 per c.c. The ratio of the average bacterial content of the former to the latter was therefore 1 : 6·3.

The higher total bacterial content of the coliform-positive samples may be due to several factors of which the following appear to be the most important: (1) a higher initial contamination of such milk not only with coliform bacteria but also with others, as a result of faulty methods and contaminated utensils; (2) the multiplication of coliform and other bacteria in milk which is not artificially cooled to a low temperature. Samples of uncooled milk not only give in many instances coliform-positive tests but they generally contain large numbers of bacteria. It will be seen from Table I that there is a high positive correlation, as would be expected, between the total bacterial content and the mean of the minimum and maximum atmospheric temperatures.

(*c*) The biological reactions of coliform bacteria isolated from milk.

Three hundred and fifty-nine strains of coliform bacteria were isolated from samples of market milk during a period extending from the middle of November to the middle of April, the "winter period," when the cows were confined to byres. Three or four strains as a rule were isolated from each sample of milk. An additional 438 strains were isolated during a period extending from the middle of June to the beginning of November, the "summer period," when the cows were at pasture for the whole or part of the day. In the latter period only one strain was isolated from each sample of milk, as it had previously been found that in most instances strains isolated from the same sample were identical in type. The total number of strains isolated was 797.

Method of isolation.

The strains were isolated from Durham's fermentation tubes containing MacConkey's bile-salt lactose peptone water, used in the routine examination of milk, which showed acid and gas production. The cultures were plated on MacConkey's neutral-red bile-salt lactose agar by the dilution plating method (2) and subcultures made on agar slopes.

Method of identification.

Stained smears were prepared from agar-slope cultures and examined microscopically to determine whether the organisms were of coliform type, *i.e.* non-sporing Gram-negative and rod-shaped. For their further identification the following tests were employed.

(1) *Fermentation of lactose, glucose, saccharose, dulcitol, adonitol, inulin, mannitol, raffinose, inositol and salicin.*

The fermentation tests were carried out at 37° C. by means of Durham's fermentation tubes, the medium consisting of 1 per cent. peptone water with Andrade's indicator and 0·5 per cent. of the fermentable substance. In the case of lactose, MacConkey's lactose bouillon was used. Incubation was continued for 14 days, and if the results were not satisfactory, the culture was replated and the identification tests repeated. All the strains isolated during the first period fermented mannitol which was therefore omitted during the second period. Only 567 strains were tested in raffinose, and owing to the cost only 479 in inositol.

(2) *Action on milk.*

Cultures in litmus milk were incubated at 37° C. to determine whether the organisms produced a permanent acid reaction and curdling within 7 days.

(3) *The methyl-red and Voges-Proskauer reactions.*

Cultures in a peptone-water medium containing 0·5 per cent. peptone, 0·5 per cent. glucose and 0·5 per cent. dipotassium hydrogen phosphate were incubated at 37° C. for 3 days, and then tested for methyl-red reaction and for the presence of acetyl-methyl-carbinol (the Voges-Proskauer reaction). In some instances where the methyl-red and Voges-Proskauer reactions did not correlate, repeat Voges-Proskauer tests were made using subcultures 24 and 48 hours old.

(4) *Production of indole.*

Cultures in peptone (Witte's or Fairchild's) water medium were incubated at 37° C. for 10 days and then tested for indole by means of Ehrlich's test.

(5) *Liquefaction of gelatin.*

Two tubes of gelatin medium were heavily inoculated from an agar-slope culture, one culture being kept at 37° C. and the other at room temperature. In the first case, the incubation was continued for 12 weeks, the culture being examined at intervals for digestion. In the second case, if the gelatin was not digested after 8 weeks at air temperature it was then incubated at 37° C., and if there was still no evidence of digestion after 4 weeks' incubation it was recorded as a non-liquefier. In examining the gelatin for liquefaction the tube was placed in cold water for a few hours to permit the gelatin if undigested by the bacteria to solidify. If the gelatin failed to solidify the tube was kept for a further twenty-four hours at room temperature before the reaction was recorded.

(6) *Ability to grow in Koser's citrate medium.*

The Koser medium employed consisted of 1·5 g. microcosmic salt, 1 g. potassium dihydrogen phosphate, 0·2 g. magnesium sulphate and 2 g. sodium citrate in 1000 c.c. distilled water(3). The tubes of medium were inoculated from agar-slope cultures, a very small quantity of inoculum being used. They were then incubated at 37° C., a positive reaction being indicated by the presence of marked turbidity within 7 days. Where the reaction was negative or where only a faint turbidity was produced, the test was repeated in duplicate.

(7) *Motility.*

Cultures in peptone-water medium were incubated at 37° C. and examined for motility at the end of 6, 8 and 24 hours. Strains which appeared to be non-motile were re-tested at least twice and in many instances four or five times, fresh cultures being prepared for this purpose. As an alternative method, the peptone-water cultures were kept at room temperature and tested for motility at 24 and 48 hours. This gave much better results than the first method, as the organisms were generally more actively motile at room temperature.

Biological characters of the strains.

All the strains isolated were Gram-negative, non-sporing and rod-shaped; capable of growing at 37° C.; fermenting glucose and lactose with formation of acid and gas; producing coagulation and permanent acidity of milk within 7 days at 37° C.

The biochemical reactions and the motility of all the strains are given in Table II. The glucose, lactose, mannitol, salicin, raffinose, and methyl-red reactions have not been included for the following reasons. In all instances the strains gave positive reactions in glucose, lactose and mannitol. In the case of salicin, 86·6 per cent. gave positive reactions and there was no definite association between the reaction and the other biochemical reactions even in strains belonging to the same species. For example, 69 of the strains belonging to the species *B. coli communis* Escherich (MacConkey's B, No. 34) (2, 4, 5) gave positive reactions in salicin and 26 gave negative; 181 of the strains belonging to the species *B. coli communior* (MacConkey's B, No. 71) gave positive reactions and 12 gave negative. The raffinose reactions are omitted as they closely resembled those with saccharose. There was a high positive association between the two reactions, the coefficient of association being +0·9998. The methyl-red reactions have not been included as there was a high negative association between the methyl-red and the Voges-Proskauer reactions, the coefficient being −0·9992. The majority of the strains which gave discordant results were indole negative and Voges-Proskauer positive. The variability of methyl-red reactions in such strains has been noted by Ruchhoft, Kallas, Chinn and Coulter (6) and others.

In regard to the other reactions which have been included in Table II, the Koser citrate reaction was positively associated with the Voges-Proskauer, the coefficient being +0·9464. A large proportion (94 out of 133) of the exceptions gave negative Voges-Proskauer and negative indole reactions. According to Koser and others, strains of this type are common in soil and water but are rare in faeces (3, 6, 7, 8, 9). The Koser reaction showed a very much higher degree of association with the indole reaction, the coefficient of association between the two reactions being −0·9905. Of the 54 exceptional strains, 23 were Koser positive, indole positive and Voges-Proskauer positive; 17 of these belonged to the species, *B. oxytocus* (MacConkey's B, No. 65) (4, 5). It is, perhaps, of interest that of 12 strains conforming to type MacConkey's B, No. 101, 10 were Koser positive, indole positive and Voges-Proskauer negative. There is therefore a very high negative association between the indole and Koser reactions of coliform bacteria, the exceptions occurring most frequently in (*a*) strains giving positive indole and positive Voges-Proskauer reactions, *e.g. B. oxytocus*, and (*b*) strains belonging to MacConkey's B, No. 101.

There was an almost complete positive association between the inositol and the adonitol reactions with the exception of a small group of strains belonging to those *B. coli* types which were Koser negative, Voges-Proskauer negative

Table II. *Showing biochemical reactions and motility of 797 strains of coliform bacteria isolated from milk.*

Group	Koser	Indole	Voges-Proskauer	Gelatin	Inositol*		Adonitol		Saccharose		Dulcitol		Inulin		Motility	
					+	−	+	−	+	−	+	−	+	−	+	−
1. *B. coli* types (432 strains)	−	+	−	−	0	200	60	372	236	196	313	119	0	432	388	44
2. *B. aerogenes* types (125 strains)	+	−	+	−	88	20	96	29	112	13	26	99	23	102	28	97
3. *B. cloacae* types (74 strains)	+	−	+	+	5	38	9	65	21	53	11	63	4	70	74	0
4. Intermediate types (87 strains)	+	−	−	−	15	42	18	69	36	51	26	61	4	83	69	18
5. Intermediate types (14 strains)	+	+	−	−	10	1	13	1	12	2	2	12	0	14	3	11
6. Intermediate types (5 strains)	+	+	+	−	6	0	5	0	5	0	1	4	2	3	0	5
7. Intermediate types (18 strains)	+	+	+	+	15	1	17	1	18	0	17	1	17	1	2	16
8. Intermediate types (7 strains)	−	−	−	−	0	6	0	7	6	1	4	3	0	7	7	0
9. Intermediate types (7 strains)	+	−	−	−	2	4	2	5	4	3	3	4	0	7	7	0
10. Intermediate types (8 strains)	−	−	+	−	3	5	3	5	5	3	3	5	2	6	4	4
11. Intermediate types (2 strains)	−	−	+	+	0	1	0	2	0	2	0	2	0	2	2	0
12. Intermediate types (13 strains)	−	+	+	−	2	11	2	11	7	6	1	12	3	10	9	4
13. Intermediate types (2 strains)	−	+	+	+	1	0	1	1	2	0	0	2	0	2	2	0
14. Intermediate types (3 strains)	−	+	−	+	0	3	0	3	2	1	0	3	0	3	3	0

* Only 479 strains tested for inositol reaction.

and adonitol positive and which like all the other Koser-negative, Voges-Proskauer-negative strains gave negative inositol reactions (see Table IV). This exceptional group included the following types of MacConkey, Nos. 1, 2 (*B. acidi lactici*), 33 and 100. The writer in more recent work has obtained similar results in an additional 120 strains isolated from bovine faeces. It has been shown by Mackie(10) that the fermentation of inositol and the formation of indole are important criteria in the grouping of coliform bacteria, and Muir and Ritchie(2) accordingly recommend their employment for this purpose. Inositol, however, owing to its cost is unsuitable for routine work. If therefore the method of classification recommended by Muir and Ritchie is used, it is possible to substitute adonitol for inositol, provided the Koser and Voges-Proskauer tests are also carried out.

The entire collection of strains is grouped in Table II according to the Koser, indole, Voges-Proskauer and gelatin reactions. The organisms of Group I are *B. coli* types. All the strains which were tested in inositol gave negative reactions. The majority of the strains in the group (86·1 per cent.) gave negative reactions in adonitol and 89·8 per cent. were motile. The saccharose and dulcitol reactions were of little significance apart from their employment in distinguishing species within the group, *e.g. B. coli communis* and *B. coli communior*. The organisms of group 2 are known as *B. lactis aerogenes* types. They differed considerably from those of group 1, not only in that they were Koser citrate positive, indole negative and Voges-Proskauer positive but also in regard to the other biochemical reactions and in the fact that the majority were non-motile. An important feature of these organisms was their power to ferment inositol(2) and adonitol; 88 out of 108 strains (81·5 per cent.) fermented the former with production of acid and gas and 76·8 per cent. fermented the latter. All the strains belonging to the species *B. lactis aerogenes* (MacConkey's B, No. 103) fermented inositol. The majority of the strains of the group (89·6 per cent.) fermented saccharose. The other reactions were of less significance in distinguishing the group. The members of group 3 are known as *B. cloacae* types. Apart from the Koser, indole and Voges-Proskauer reactions, they resembled more closely the organisms of group 1 than those of group 2. In all instances the strains isolated were motile and the majority gave negative reactions in inositol and adonitol. The remaining groups, Nos. 4–14, were intermediate in type in regard to Koser, indole, Voges-Proskauer and gelatin reactions, but in regard to other biochemical reactions and motility, Nos. 5, 6 and 7, were very similar to group 2, while the others resembled closely group 1. The members of group 6 may be regarded as indole-forming *B. lactis aerogenes* types, but those of group 7 cannot be regarded as indole-forming *B. cloacae* types, as in their inositol and adonitol reactions, and as regards motility, they resembled closely the *B. lactis aerogenes* types. The members of group 8 may be regarded as indole-negative *B. coli* types(6). The number of strains isolated in each of the intermediate groups was too small for any conclusion to be drawn in regard to the significance of the biochemical reactions.

It will be seen from Table III that 54·2 per cent. of the entire collection of strains belonged to group 1 (*B. coli* types), 15·7 per cent. to group 2 (*B. lactis aerogenes* types), 9·3 per cent. to group 3 (*B. cloacae* types), and 10·9 per cent. to group 4 (intermediate types). Further, there was a much higher proportion of the *B. coli* strains (group 1) isolated from milk during the winter period than during the summer, 71·0 per cent. as compared with 40·4 per cent., but a much lower proportion of the *B. lactis aerogenes* strains

Table III. *Strains of coliform bacteria found in milk during the winter period (when cows were not at pasture) and during the summer period (when cows were at pasture).*

	Number of strains isolated					
	Winter period		Summer period		Combined periods	
Group (see Table II)	Total	%	Total	%	Total	%
1. *B. coli* types	255	71·0	177	40·4	432	54·2
2. *B. aerogenes* types	27	7·5	98	22·4	125	15·7
3. *B. cloacae* types	31	8·6	43	9·8	74	9·3
4. Intermediate types	28	7·8	59	13·5	87	10·9
5. ,,	6	1·7	8	1·8	14	1·8
6. ,,	1	0·3	4	0·9	5	0·6
7. ,,	3	0·8	15	3·4	18	2·3
8. ,,	3	0·8	4	0·9	7	0·9
9. ,,	0	.	7	1·6	7	0·9
10. ,,	0	.	8	1·8	8	1·0
11. ,,	0	.	2	0·5	2	0·3
12. ,,	4	1·1	9	2·1	13	1·6
13. ,,	1	0·3	1	0·2	2	0·3
14. ,,	0	.	3	0·7	3	0·4
	359		438		797	

Table IV. *Showing the positive association that occurs between the inositol and adonitol reactions in strains of coliform bacteria with the exception of strains which are Koser negative, Voges-Proskauer negative and adonitol positive.*

			Inositol Number of strains	
Koser	Voges-Proskauer	Adonitol	+	−
−	−	+	0	34
−	−	−	0	175
−	+	−	0	17
−	+	+	6	0
+	−	−	0	46
+	−	+	27	1
+	+	−	6	57
+	+	+	108	2

(group 2) isolated during the winter than during the summer, 7·5 per cent. as compared with 22·4 per cent. The proportion of *B. cloacae* types (group 3) was approximately the same for both periods. (Of all the strains isolated, 36·1 per cent. belonged to the species *B. coli communis* and *B. coli communior*, both characteristic faecal types belonging to group 1.) It may therefore be said that in the winter period 71·0 per cent. of the strains isolated were of the types most prevalent in faeces (the *B. coli* types), while in the summer period only 40·4 per cent. were of these types.

Discussion.

In regard to the results of the present investigation the following points require to be discussed: (1) the effect of atmospheric temperature on the proportion of coliform-positive samples and on the total bacterial content of mixed cow's milk; (2) the significance of different types of coliform bacteria in milk.

(1) The positive correlation that has been found to occur between the proportion of coliform-positive samples, the average total bacterial content and the atmospheric temperature appears to be due to the influence of higher temperatures both on milk which is not artificially cooled and on incompletely sterilised utensils, so that more rapid multiplication of contaminating organisms occurs. It must be borne in mind, however, that although the samples were taken from mixed cow's milk under fair conditions of cleanliness and were typical of the general supplies from the farms, the conditions at milking in many cases might vary to some extent according to season. During winter the cows were confined to the byres and were supplied with food and bedding, while in the summer they were at pasture and were given little or no house-feeding and no bedding. Consequently in winter the atmosphere of the byres would generally be contaminated to a greater extent with particles of faeces, food and bedding. This would increase the contamination of the milk during winter with coliform and other bacteria and render less apparent the correlation that occurs between bacterial content, prevalence of coliform organisms and atmospheric temperature. On the other hand, during wet weather in summer milk may be highly contaminated owing to cows at pasture becoming heavily soiled with mud and faeces from fields and gateways. Further, cows at pasture, especially in early summer, suffer to a greater extent from diarrhoeal conditions than those confined to houses. These two factors might be partly responsible for the higher total bacterial content and higher incidence of coliform organisms in milk during the summer.

(2) In considering the significance of the occurrence of different types of coliform bacteria in milk the question of their origin is of importance. It is well known that these organisms occur in large numbers in the human and animal intestines and in faeces, and in smaller numbers in the soil and on cereal grains and plants. There is still, however, a difference of opinion as to the distribution of the various types in nature and their occurrence in faeces. As a result of the work of numerous investigators (3–9; 11–21) the following facts have been established: (1) Bacteria of the *B. coli* type (group 1) are the prevalent coliform organisms in human and animal faeces, and while they are frequently found in surface waters and the soil they are present in such environment only in very small numbers provided recent contamination with faeces has not occurred. (2) *B. lactis aerogenes* and *B. cloacae* types (groups 2 and 3) are the predominating coliform types in the soil and on plants and grain and in surface water provided there has been no recent pollution with

faeces. The *B. coli* types have therefore been commonly regarded as faecal organisms and the *B. lactis aerogenes* and *B. cloacae* types as non-faecal. There is, however, evidence in the work of numerous investigators to show that the *B. lactis aerogenes* and *B. cloacae* types are not confined to the soil and surface waters but also occur in human and animal intestines and in faeces, though in comparatively small numbers. Ruchhoft, Kallas, Chinn and Coulter (6) found that it was possible to obtain such types from faeces provided special enrichment methods were used to isolate them from the more prevalent *B. coli* types. They attributed the failure of other investigators to obtain them from faeces to their omission to employ such methods. Cruickshank (22) found that such organisms occurred in small numbers in the faeces of the majority of normal people, and Hay (23) showed that they were more abundant in diarrhoeal conditions. Recent work by the writer has indicated that they can be isolated from time to time from bovine faeces especially if an enrichment method is employed, *e.g.* by using Koser's citrate medium. It would therefore appear that the *B. lactis aerogenes* and *B. cloacae* types occur frequently in human and animal faeces but in small numbers in health. If Hay's results in regard to diarrhoeal conditions apply also to bovine faeces, then the greater incidence of *B. lactis aerogenes* types in milk from cows at pasture might thus be explained, as the animals under such conditions are more liable to suffer from diarrhoea. At present it is impossible to say much about the source of the intermediate types (groups 4–14) with the exception of the members of groups 4 and 5, which have been assigned by Koser and Ruchhoft, Kallas, Chinn and Coulter to the soil (3, 6).

The occurrence of *B. lactis aerogenes* and *B. cloacae* types in faeces raises the question as to the differentiation of faecal and non-faecal types of coliform organisms. The problem is rendered more difficult owing to the existence of intermediate types. All that can be said at present is that certain types predominate in faeces, others in the soil. The former in most instances form indole, give negative Voges-Proskauer reactions and fail to grow in Koser's citrate medium. On the other hand, the majority of those types that are prevalent in the soil fail to form indole, give positive Voges-Proskauer reactions and grow in Koser's medium. In regard to the occurrence of coliform bacteria in milk one might say that the typical *B. coli* are derived from faeces and their presence in milk may be taken as a reliable indication of faecal contamination, but since the origin of *B. lactis aerogenes*, *B. cloacae* and the intermediate types is still uncertain, the presence of these in milk is of no clear significance. Further, one cannot suggest an explanation of the much greater incidence of the latter types in milk of cows at pasture until the source of these organisms is definitely established. This is forming the subject of further investigation.

SUMMARY.

1. Of 21,569 samples of mixed cow's milk taken under fair conditions of cleanliness and examined by means of the coliform test, 48·3 per cent. gave negative results with 1/10, 1/100 and 1/1000 c.c. amounts; 21·4 per cent. gave positive results with only 1/10 c.c.; 14·0 per cent. gave positive results with only 1/10 and 1/100 c.c.; and 16·3 per cent. gave positive results with 1/10, 1/100 and 1/1000 c.c. It is probable that these results are better than those generally obtained.

2. The proportion of coliform-positive samples was much higher in summer and early autumn than during the winter and spring. This seasonal variation was largely due to atmospheric temperature, there being a well-marked direct correlation between the proportion of coliform-positive samples and the mean of the minimum and maximum atmospheric temperatures.

3. Of 21,857 samples examined, 10,458 were coliform negative and had an average bacterial content of 25,294 per c.c.; 11,399 were coliform positive and had an average bacterial content of 160,577 per c.c. The coliform-positive samples contained on an average 6·3 times as many bacteria as the coliform negative.

4. There is a well-marked positive correlation between the average bacterial content of a series of samples, the proportion of coliform-positive samples and the mean of the minimum and maximum atmospheric temperatures.

5. Seven hundred and ninety-seven strains of coliform bacteria were isolated from samples of milk, and the degrees of association between certain characters examined with the following results:

(*a*) The coefficient of association between the fermentation of saccharose and of raffinose was +0·9998, *therefore one or other of these tests may be omitted.*

(*b*) The coefficient of association between the methyl-red and Voges-Proskauer reactions was −0·9992. The exceptions in most instances were strains of the *B. lactis aerogenes* and *B. cloacae* types.

(*c*) There was a positive association between the Koser citrate and the Voges-Proskauer reactions, the coefficient of association being +0·9464. Exceptions occurred most frequently in strains which were indole negative and Voges-Proskauer negative or indole positive and Voges-Proskauer positive. There was a very high negative association between the Koser citrate and indole reactions, the coefficient being −0·9905. The exceptions occurred most frequently in MacConkey's B, No. 101 and in types giving indole positive and Voges-Proskauer positive reactions, especially *B. oxytocus* (MacConkey's B, No. 65).

(*d*) There was an almost complete positive association between the inositol and the adonitol reactions with the exception of a small group of *B. coli* types which were Koser negative, Voges-Proskauer negative and adonitol positive and which, like all the other Koser negative, Voges-Proskauer negative types,

gave negative inositol reactions. This small exceptional group included the following types: MacConkey's B, Nos. 1, 2 (*B. acidi lactici*), 33 and 100.

6. Of the strains isolated from samples of milk during the colder part of the year (winter and spring) when the cows were confined to the byres, 71·0 per cent. were of the types which predominate in faeces (*B. coli* types), 7·5 per cent. were of the *B. lactis aerogenes* types, 8·6 per cent. of the *B. cloacae* types, and there were 7·8 per cent. of an intermediate type which was Koser positive, indole negative, methyl-red positive, Voges-Proskauer negative. Of the strains isolated from samples of milk during the warmer part of the year (summer and autumn) when the cows were at pasture, 40·4 per cent. were of the *B. coli* types, 22·4 per cent. of the *B. lactis aerogenes* types, 9·8 per cent. of the *B. cloacae* types and 13·5 per cent. of the aforementioned intermediate type. There was therefore a much higher proportion of typical *B. coli* strains isolated when the cows were confined to the byres than when they were at pasture. The species of coliform bacteria most frequently found were (in order of frequency) *B. coli communior*, *B. coli communis*, *B. cloacae* and *B. lactis aerogenes*.

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69. THE BURRI SMEAR-CULTURE METHOD FOR THE DETERMINATION OF THE BACTERIAL CONTENT OF MILK SAMPLES

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At a meeting of the Eighth International Dairy Congress held in Reading in July, 1928, Burri(3) contributed a paper on a new method for estimating the bacterial content of milk. In determinations made by this method small volumes of the sample, measured by means of platinum loops, are spread over the surfaces of agar slopes, the cultures are incubated at a suitable temperature, the colonies which develop are counted and the bacterial content per c.c. of the sample is calculated. Burri recommended that loops of 1 c.mm. capacity should be employed and that the agar slopes should be dried at room temperature for 3–6 weeks before use. He considered the method to be specially valuable for the examination of samples of low germ content such as graded milks, because bacterial numbers of from 1000 to 100,000 per c.c. can be dealt with in cultures inoculated with measured volumes of the undiluted sample. In cases in which values in excess of 100,000 per c.c. are anticipated a 1 in 100 dilution of the milk is prepared and examined as already described, while for the examination of samples drawn aseptically it is suggested that five loopfuls of the undiluted milk should be spread over the surface of each of two agar slopes.

For the regular bacteriological control of milk samples it is claimed that Burri's method possesses the following advantages:

(1) The technique involved is extremely simple.

(2) The results obtained are generally higher than those secured by the use of the ordinary pipette-plate method.

(3) As compared with the plate method there is considerable economy of time and material.

(4) In the inoculation of slopes there is less risk of contamination from outside sources than in the inoculation of plates.

(5) Slopes do not dry so readily as plates, and therefore, they can be kept longer.

(6) Colonies of individual micro-organisms on slopes are uniform in appearance and generally more characteristic than the deep colonies in plates; slope cultures are, therefore, more valuable than plates for qualitative study.

(7) Determinations of bacterial content by the Burri method can be made in any part of a dairy or milk plant.

Since 1928 a number of papers dealing with the Burri technique has been published. Dorner(6) used the method in a series of comparisons with the

plate method for the quantitative and qualitative bacteriological examination of a large number of samples of aseptically drawn milk. He claims that it gives higher results than plating and that it permits a considerable development of organisms similar to *Bact. lipolyticum* Evans, which are present in small numbers only on ordinary plates. Dorner, therefore, recommends the Burri technique in preference to the plate method for the examination of samples of Certified and Grade A raw milk. Richter(10) compared the loop method of Burri with ordinary pipette-plating and with a number of the other methods commonly employed in the examination of milk. He considers that the Burri method yields useful results when the milk is diluted so that not more than 150 colonies develop on each slope, but he reaches the conclusion that it is less reliable than the pipette-plate technique. As Dorner and Demont(7) have pointed out, however, it is unjustifiable to take the plate method as a standard, as Richter has done, and to attribute all deviations of the results secured by the smear-culture method from those obtained by plating to the unreliability of the Burri technique. Demont and Dorner(5) recommended peptone whey agar as a good, cheap and simple medium for use in the smear-culture method, while Dorner and Demont(7) made a study of the accuracy and limitations of the latter. These workers claim that the Burri technique gives higher values than the plate method to which it is not inferior in point of accuracy.

The advantages which have been claimed for it appear to indicate that the smear-culture method might prove to be of value in advisory work where large numbers of milk samples frequently require to be examined in a comparatively short time. An investigation of the accuracy of the method when compared with that of the ordinary pipette-plate technique was, therefore, carried out.

Methods.

Throughout the work described in this paper, except where otherwise stated, the medium employed had the following composition:

Meat extract ("Lab Lemco")	3 g.
Peptone ("Difco Bacto")	5 g.
Agar	15 g.
Water...	1000 c.c.
*p*H adjusted to	7·0

The agar for plate cultures was tubed in approximately 8 c.c. quantities, while for smear-cultures each tube ($6 \times \frac{5}{8}$ in.) contained about 4 c.c. of the medium solidified in a sloped position. The slopes were dried by placing them in an incubator at 37° C. for 3–4 days. Determinations of the bacterial content of milk samples by both the pipette and loop techniques were made in duplicate, except where a statement to the contrary appears in the description of the experiments. Comparative tests were carried out with medium of the same batch.

In the case of the plate method each Petri dish received an inoculation of 1 c.c. of a suitable dilution of the milk, and all inoculations were made by means of sterile pipettes which had been calibrated. The milk samples were diluted in narrow glass-stoppered bottles (1½ in. internal diameter) with horizontal marks at the 100 c.c. levels, and each dilution was prepared by transferring 10 c.c. of the milk or of the previous dilution to a sterile bottle and adjusting the level of the liquid to the mark by adding sterile water.

When loops were employed for the measurement of milk or of dilutions to be used for the inoculation of media, the loop was always inserted into the liquid until it was just covered, and in all cases the wire bearing the loop was held vertically during the insertion and withdrawal of the latter. Dorner and Demont (7) recommend that the loop should make an angle of 45° with the surface when the measured volumes of liquid for inoculation are being removed from the bulk. For the following reasons, however, it appeared that it would be possible to reproduce results with greater accuracy when the wire was held vertically than when it made an angle of 45° with the surface of the liquid. In transferring liquid cultures, it is a common experience that, as the inoculating wire approaches the horizontal, the volumes of liquid taken up by loops are liable to show extreme variations. It is also less difficult to judge when a needle is vertical than it is to be certain that it makes an angle of 45° with the surface of the liquid from which the inoculum is taken. In this connection it may be mentioned that Miller (8), who made a careful study of loop measurements, recommends that the plane of the loop should be held vertically. Breed and Brew (1), in the description of their work on this subject, also state that the loops were withdrawn vertically from the liquid.

By means of an occasional treatment with concentrated sulphuric acid, the platinum loops employed in this work have been maintained in a satisfactory condition. They have remained clean and bright, and during a period of many months have shown little or no tendency to exhibit an uneven surface. Except where it is otherwise stated, the contents of the loop or loops used were spread as evenly as possible over the entire surface of the medium.

All cultures were incubated at 37° C., the Burri and Petri dish cultures belonging to each experiment being kept together in the same incubator. The colonies were counted after 48 hours' incubation. Neither the ordinary plates nor the smear-cultures showed an appreciable increase in numbers of colonies when incubation was prolonged to 72 hours. In the case of the slopes, however, the colonies tended to grow larger and to coalesce, thus causing an apparent lowering of the results obtained on enumeration. From the point of view of ease and accuracy, it was found to be advisable to count the colonies on slopes immediately after these had been removed from the incubator and before condensation of moisture on the surface of the glass had taken place.

Experimental.

A considerable number of preliminary experiments was carried out in order to obtain approximate data on the magnitude of the errors involved in duplicate determinations made by the Burri and ordinary pipette-plate techniques. In a few of these experiments a 2 c.mm. resistance-wire loop was employed, but the major part of the work was done with a 1 c.mm. platinum loop. The latter was obtained from the source recommended by Burri (A. Stoppani A.-G., Werkstätten f. Präzisionsmechanik, Bern); the former was made in the laboratory. Duplicate plate determinations were compared with the corresponding values obtained by the smear-culture technique, in which one or two loopfuls of milk or of a suitable dilution were used for the inoculation of each slope. The results of these experiments indicated that agreement between duplicates prepared by Burri's method was inferior to that of parallel plates inoculated by means of pipettes, and that in the case of certain samples duplicate slopes showed agreement which was extremely unsatisfactory. It appears unnecessary to refer to the results of these experiments in detail. In order to indicate roughly how the agreement of duplicate determinations made by the two methods compared, it may suffice if the number of cases in which the higher result of a pair differed from the lower by 100 per cent. or more are quoted. For convenience, such results will be referred to as "erratic." It should be noted, however, that the figures given do not adequately represent the superiority of the plate over the smear-culture method. In the majority of the duplicate plates agreement was excellent, whereas many of the duplicate slopes, although they did not exhibit erratic behaviour in the sense defined above, were markedly less uniform than the corresponding plate cultures. Ninety-three samples of milk were examined by both methods. From certain samples more than one pair of slopes was prepared, so that a total of 93 pairs of plates and 131 pairs of slopes was examined. None of the duplicate plates showed erratic results, whereas 24 of the pairs of slope cultures were definitely erratic in behaviour. Further preliminary experiments were carried out with the object of determining whether duplicate agreement in smear-cultures could be improved by the introduction of certain modifications in technique. The alterations made included the use of a new platinum loop for inoculation, variations in the surface areas inoculated and substitution of ordinary meat extract, meat infusion and glucose agars for the medium originally used. None of the modifications introduced, however, effected an appreciable improvement in the agreement of the results from duplicate slopes.

A study of the literature shows that there is some diversity of opinion as to the value of metal loops for the accurate measurement of small volumes of liquid. In determinations of the bacterial content of milk, Conn (4) is reported to have found loops as accurate as pipettes. Miller (8) determined the capacities of loops by application of capillaries to the edges of the filled loops and measurement of the liquid columns in the tubes. In three series of ex-

periments the maximum deviations were 2·8, 2·9 and 4·6 per cent. In his description of the smear-culture technique Burri (3) stated that weighings of the contents of loops holding 0·5–1·0 mg. seldom differed by more than 5 and never by more than 10 per cent. On the other hand, Breed and Brew (1) showed by means of weighings that calibrated platinum loops of capacity 0·01 c.c. have a maximum deviation of 35 per cent., while Davis quotes Peskett (9) as stating that the maximum deviation of calibrated loops may amount to 55 per cent.

In view of the conflicting opinions of previous workers regarding the errors involved in loop measurements and also of the results obtained in the preliminary experiments, it appeared advisable to test the accuracy of the loops used in this investigation. For this purpose the procedure adopted was as follows: 1 c.c. of sterile water was placed in each of a series of Petri dishes, and two loopfuls of the milk sample to be examined were transferred to each

Table I. *Comparison of the pipette-plate and loop-plate methods for the determination of the bacterial content of milk samples.*

		Pipette-plate method			Loop-plate method		
		Number of colonies per plate			Number of colonies per plate		
No. of exp.	Volume of milk examined c.c.	(*a*)	(*b*)	Bacterial content per c.c.	(*a*)	(*b*)	Bacterial content per c.c.
1	0·01	200	190	19,500	45	72	29,250
2	0·01	144	118	13,100	60	16	19,000
3	0·01	63	65	6,400	28	26	13,500
4	0·001	220	205	212,500	98	101	50,000
5	0·001	34	36	35,000	54	55	27,250
6	0·001	16	24	20,000	35	27	15,500
7	0·01	160	140	15,000	35	33	17,000
8	0·01	200	180	19,000	36	57	23,250
9	0·001	150	170	160,000	86	90	44,000
10	0·01	206	208	20,700	30	28	14,500
11	0·001	210	250	230,000	128	164	73,000
12	0·001	200	240	220,000	92	100	48,000

of two dishes, the contents of the loops being deposited on the glass and the loops themselves thoroughly washed in the water. In this and subsequent work, only platinum loops obtained from Stoppani were employed. After the Petri dishes had been inoculated a tube of melted agar at 45–50° C. was poured into each dish, mixed with the water and allowed to solidify. The plates were incubated as already described and the colonies counted. The bacterial content of the milk samples was also determined by ordinary pipette-plating: the volumes of milk used for the inoculation of each plate are shown in Table I, which gives the results of twelve of the experiments. Excluding tests in which the mean number of colonies per plate in one or both determinations was less than 20 or more than 400, twenty-nine samples were examined by these methods; Table I shows the values obtained in the first six and in the last six experiments, which are fairly representative of the whole series. The agreement in the results from duplicate cultures inoculated by means of loops is more satisfactory than that observed in the preliminary experiments. In

certain samples, however, it is still inferior to that of plates inoculated by means of pipettes. Only two erratic results were encountered in the twenty-nine sets of determinations, but both were in measurements made by means of loops. It may also be observed that there is an almost complete lack of agreement between the mean values obtained by the two methods for the bacterial content of the samples examined. The results demonstrate that measurements of small volumes of liquids made by means of loops employed as in these experiments are frequently unreliable.

It appeared probable that 2 c.mm. was too small a volume of liquid to permit of accurate measurement by the means available and that if the inoculum was increased more accurate results might be obtained. A further series of experiments was, therefore, carried out with the object of determining whether greater accuracy could be attained by the use of ten loopfuls per

Table II. *Comparison of the pipette-plate and loop-plate methods for the determination of the bacterial content of milk samples.*

	Pipette-plate method			Loop-plate method		
	Number of colonies per plate			Number of colonies per plate		
No. of exp.	(*a*)	(*b*)	Bacterial content per c.c.	(*a*)	(*b*)	Bacterial content per c.c.
1	80	76	7,800	85	90	8,750 (1)
2	180	190	18,500	180	186	18,300 (1)
				180	190	18,500 (1)
3	288	310	29,900	295	320	30,750 (1)
				300	330	31,500 (1)
4	94	110	10,200	100	112	10,600 (1)
				93	108	10,050 (2)
				102	115	10,850 (3)
5	80	90	8,500	93	98	9,550 (1)
				92	100	9,600 (2)
				98	106	10,200 (3)
6	200	220	21,000	230	250	24,000 (1)
				240	270	25,500 (2)
				250	260	25,500 (3)

(1) = loop No. 1; (2) = loop No. 2; (3) = loop No. 3.

culture. The arrangement of the experiments was similar to that of the first series with the exceptions that in the pipette-plate method 1 c.c. of the 1 in 100 dilution of the milk was used for the inoculation of each Petri dish, while in the loop technique the inoculum for each culture consisted of ten loopfuls of the milk. Table II shows the results. In the case of samples 1–3 the same loop was employed throughout, while for samples 4–6 three loops were used and separate determinations were made on each of the samples by means of each loop. The figures indicate that in the experiments in which the loop method was used agreement between duplicates was as satisfactory as in those in which the measurements were made by pipettes. The values obtained by the two methods for the bacterial content of the milk samples also showed satisfactory agreement except in the cases of samples 1, 5 and 6. For samples 5 and 6 all three loops yielded decidedly higher results than the pipettes. In view of this fact, it seems probable that the lack of agreement

between the final results obtained by the two methods for samples 5 and 6 is to be attributed mainly to some special characteristic of the samples themselves.

The data in Table II demonstrate that in determinations of the bacterial content of milk samples made by the loop-plate method it is possible to obtain satisfactory agreement between parallel results when ten loopfuls of liquid are taken for each determination. The next step in the investigation, therefore, was to compare the pipette-plate technique with the Burri method in which each slope received an inoculation of ten loopfuls of liquid. In order that the tests might be applied under conditions as favourable as possible for the smear-culture method, in all cases the slopes were inoculated from a 1 in 10 dilution of the milk sample, thus reducing the effect of possible disturbing influences due to special characteristics of the individual milk samples. Throughout this section of the work loops 2 and 3 (Table II) were employed. They were mounted side by side in one holder, in order to reduce the labour involved in the inoculations. In the pipette-plate method each Petri dish was inoculated with 1 c.c. of a 1 in 1000 dilution of the sample. The Burri and plate techniques were as described under "Methods" except that ten parallel slopes and plates were prepared from each sample. The latter modification was introduced with the object of rendering possible the application of statistical methods in the interpretation of the results. In Table III the results for the fifty samples examined have been arranged in the numerical order of the arithmetic means for the slope cultures. The standard errors were calculated from the expression $\sqrt{\frac{s(x-\bar{x})^2}{n(n-1)}}$, in which $s(x-\bar{x})^2$ = the sum of the squares of the deviations and n = the number of parallel determinations. Under "Percentage error" are shown the values for the standard errors expressed as percentages of the arithmetic means.

A study of Table III indicates that for the slopes the percentage errors are in general considerably higher than for the plates: the values for the plate technique vary from 1·55 to 9·80, while those for the smear-culture method vary from 4·32 to 27·24. In three cases only are the figures for the plates higher than the corresponding values for the slopes, and in all three the differences are slight. An approximate estimate of the errors involved in the two methods may be obtained by calculating the arithmetic means of the percentage errors. The values referred to are $4{\cdot}08 \pm 2{\cdot}24$ for the plate method and $9{\cdot}23 \pm 3{\cdot}90$ for the Burri method. Dorner and Demont(7) have advanced evidence in support of the view that on Burri slopes the permissible limits in numbers of colonies per slope are 25 and 100. In the present investigation thirty-two samples (Nos. 2–33 inclusive) fall between these limits; in the thirty-two samples in question the percentage error shows a variation of from 1·68 to 9·80 for the plates and 5·77 to 27·24 for the slopes, while the arithmetic means of the percentage errors are $4{\cdot}94 \pm 2{\cdot}34$ and $9{\cdot}90 \pm 4{\cdot}25$ respectively.

It is generally recognised that, although the medium employed in these

studies has been selected as that to be used in official determinations of the bacterial content of graded milks, it is not entirely satisfactory, as many

Table III. *Comparison of the percentage errors in determinations made by the ordinary plate and Burri slope methods.*

Sample No.	Arithmetic mean		Percentage error	
	Plates	Slopes	Plates	Slopes
1	21·1	21·8	5·07	11·72
2	16·9	26·6	4·94	14·69
3	38·5	26·8	9·24	7·71
4	40·9	30·7	4·12	10·49
5	14·7	39·1	9·80	27·24
6	24·2	40·2	4·47	7·69
7	38·0	46·9	4·45	8·39
8	19·0	48·4	4·23	12·96
9	33·1	52·6	5·86	9·76
10	64·3	56·5	7·13	8·97
11	93·1	61·0	3·53	6·83
12	124·9	62·4	1·68	10·60
13	86·2	63·2	1·69	8·32
14	64·2	63·8	5·35	6·64
15	114·0	68·1	2·93	6·04
16	54·1	68·6	1·79	9·78
17	63·7	69·6	3·13	8·82
18	40·5	71·0	3·20	11·76
19	98·4	71·8	7·62	6·12
20	85·4	73·8	2·80	8·03
21	62·3	74·4	3·98	8·52
22	88·9	76·9	3·94	9·33
23	71·2	77·7	6·72	12·60
24	42·6	78·6	8·00	10·14
25	137·2	81·4	1·76	8·43
26	23·8	85·2	7·54	19·40
27	70·9	86·4	2·18	6·05
28	136·7	87·1	3·36	5·77
29	137·0	87·7	8·42	6·99
30	57·6	89·4	4·34	7·12
31	56·4	90·6	8·04	10·52
32	84·9	92·6	6·12	11·76
33	112·9	96·4	5·70	9·48
34	125·1	103·4	2·01	6·67
35	118·1	107·2	1·85	11·83
36	42·9	107·3	1·85	10·05
37	55·4	109·2	2·90	15·24
38	123·0	114·8	3·14	7·44
39	81·8	115·2	2·93	5·66
40	136·8	118·4	2·32	4·32
41	88·6	122·3	2·61	9·18
42	120·8	127·4	2·60	4·45
43	94·6	141·4	2·52	6·98
44	187·1	148·4	1·55	6·75
45	87·7	149·0	2·46	9·44
46	178·5	149·8	2·19	10·42
47	131·0	166·8	2·61	7·21
48	220·4	167·5	2·33	5·94
49	173·1	174·2	2·83	5·76
50	127·7	176·9	1·92	5·70
		Mean (all samples)	4·08 ± 2·24	9·23 ± 3·90
		Mean (samples 2–33)	4·94 ± 2·34	9·90 ± 4·25

characteristic milk organisms develop poorly upon it. It appeared to be advisable to determine whether the inferiority of the smear-culture to the plating technique, as demonstrated by the results already quoted, could be

eliminated by the use of a better medium. Thirteen additional samples were examined by the Burri and plating methods already described, the media employed being glucose agar and milk agar prepared by the addition of 1 per cent. of skim milk to agar of the same composition as that used in the previous tests. The results of these experiments, however, showed that no appreciable improvement in the agreement between duplicate smear-cultures could be brought about by the modifications described. The whole of the evidence obtained in these studies, therefore, indicates that the Burri technique is decidedly less accurate than the pipette-plate method.

It has been noted that Dorner and Demont(7) reached the general conclusion that the Burri technique is not inferior to the pipette method in point of accuracy—a result at variance with the findings in this investigation. Attention is directed to the fact that while these studies were carried out under conditions which favoured the loop technique in that ten loopfuls of liquid were employed for the inoculation of each slope, Dorner and Demont appear to have employed a single loopful for the inoculation of each Burri culture. The lack of agreement in the results of the two investigations is, therefore, the more surprising.

Dorner and Demont's conclusions are based upon twenty-nine comparisons of the two methods. For their experiments the following materials were employed:

3 samples of normal milk for 5 comparative tests;

7 samples of homogenised milk for 7 comparative tests;

3 bouillon cultures of *Streptococcus lactis* for 17 comparative tests.

Reference has already been made to Dorner and Demont's conclusion that the Burri method yields unreliable results when the number of colonies per slope exceeds 100 or falls below 25. As the mean number of colonies per tube or plate falls below about 30, the coefficient of variation is shown to increase rapidly in both methods. It is inadvisable, therefore, to base conclusions as to the variability of parallel determinations on results obtained under such conditions. On the other hand, as the number of colonies increases beyond 100 the coefficient of variation is slowly reduced. Another factor, however, which affects the reliability of the results comes into play; the development of the colonies is retarded or inhibited owing to overcrowding, and the lower values for the coefficient of variation are to be attributed at least partially to this influence. Differences between parallel determinations are reduced owing to the fact that the development of colonies is restricted. On plates the point at which this influence begins to operate is considerably higher than 100 colonies per plate: many workers consider that plates each containing from 300 to 400 colonies can be utilised for making accurate determinations of bacterial content by the usual methods(2). Owing to the fact that development of colonies on plates does not become restricted until about 400 colonies are present on each plate whereas, according to Dorner and Demont, 100 colonies per slope is the maximum permissible, it is clear that the comparison

of the variability of results obtained from slopes showing more than 100 colonies each with that of values from plates containing about the same numbers tends unduly to favour the Burri method.

In a considerable number of Dorner and Demont's experiments the mean values for the determinations made by Burri's method are either lower than 25 or higher than 100. If experiments containing results in these categories are excluded, the numbers of comparative tests are reduced to:

Normal milk	1
Homogenised milk	3
Streptococcus lactis cultures	6

In the experiments referred to, the extreme coefficients of variation are 7·6 and 23·9 in the plate cultures and 15·4 and 37·8 in the slopes; in eight cases out of ten the data show that the Burri method was less reliable than the plate technique. It is doubtful, however, whether the values for homogenised milk samples and for *Streptococcus lactis* cultures can be assumed to be applicable to samples of normal milk. Homogenisation might be expected to lower the variability of parallel determinations made by both methods, but whether it would influence both series of results in a similar manner cannot be predicted. Further, results obtained from bouillon cultures of *Streptococcus lactis* are not necessarily applicable to milk; in our experience pure strains of milk streptococci have behaved erratically in both plate and slope cultures. In the case of the sample of normal milk, in which the mean number of colonies per plate was only 12·5, the coefficients of variation were 19·3 for the plates and 37·8 for the slopes.

Dorner(6) and Dorner and Demont(7) have claimed that the Burri method yields higher results than the pipette-plate technique. A study of the arithmetic means of the results obtained by the two methods (Table III) affords no support to this claim. In view of the variability of parallel determinations made by Burri's method as demonstrated in the present investigation it is questionable whether one is justified in attempting a strict comparison.

In order to obtain further information concerning the causes of the variability of duplicate determinations made by means of smear-cultures, two additional series of experiments were carried out. In the first of these the general arrangement of the tests was similar to that of the experiments, the results of which are reported in Table III, except that a second set of plate cultures was added. Ten plates were prepared, allowed to solidify and dried for 24 hours in the 37° C. incubator. They were then inoculated in a manner similar to that employed in the inoculation of the slopes, care being taken to spread the inoculum as evenly as possible over the agar without breaking the surface of the latter. After incubation the colonies on the three sets of cultures were counted and the percentage errors calculated. The results for those experiments in which the arithmetic means for the slopes were between 25 and 125 are shown in Table IV. The arithmetic means of the percentage errors are, for the plate method $4{\cdot}65 \pm 3{\cdot}00$, for the Burri slope method $9{\cdot}65 \pm 6{\cdot}32$,

and for the Burri plate method 9·74 ± 6·48. The means for the two sets of Burri cultures are of the same order of magnitude and are considerably higher than the corresponding results for the plate cultures. In these experiments the mean percentage errors for the plate and Burri cultures approach closely to the values for plate and slope cultures obtained in the experiments, the results of which are recorded in Table III. The substitution of plates for slopes in the smear-culture technique, therefore, does not reduce the liability to variation of parallel determinations made by this method.

Table IV. *Comparison of the percentage errors in determinations made by the ordinary plate, Burri slope and Burri plate methods.*

No. of exp.	Plate method	Burri slope method	Burri plate method
1	1·79	9·78	6·82
2	9·80	27·24	21·31
3	7·62	6·12	4·10
4	8·42	6·99	5·03
5	3·98	8·52	6·85
6	1·69	8·32	4·52
7	5·35	6·64	7·46
8	2·93	5·66	6·92
9	3·13	8·82	20·51
10	1·76	8·43	13·89
Mean	4·65 ± 3·00	9·65 ± 6·32	9·74 ± 6·48

The general arrangement of the second series of experiments was similar to that of the first except that after the inoculation of the Burri plate cultures a tube of melted agar at 45–50° C. was poured over the surface of the solid medium in each Petri dish and allowed to solidify. The cultures were incubated with the Petri dishes and slopes belonging to the other two sets, the colonies were counted, and the percentage errors were calculated as already described.

The results of those experiments in which the arithmetic means for the slopes were between 25 and 125 are shown in Table V. All the evidence

Table V. *Comparison of the percentage errors in determinations made by the ordinary plate, Burri slope and modified plate methods.*

No. of exp.	Plate method	Burri slope method	Modified plate method
1	2·18	6·05	3·04
2	3·94	9·33	6·76
3	7·54	19·40	4·74
4	4·45	8·39	4·96
5	4·47	7·69	4·22
6	8·00	10·14	5·00
7	1·85	10·50	2·50
8	8·04	10·52	8·68
9	2·90	15·24	6·68
10	6·72	12·60	4·45
11	3·20	11·76	8·83
12	2·61	9·18	4·94
Mean	4·66 ± 2·32	10·90 ± 3·58	5·40 ± 1·98

obtained in the preliminary tests and in the previous series of experiments indicates that in inoculations made on the surface of agar by means of loopfuls of liquid the area over which the inoculum is spread (within reasonable limits) and the type of culture vessel used exert no appreciable influence upon the

agreement between duplicate determinations. In the set of plate cultures prepared by the modified method, therefore, the inoculations were made under conditions similar to those prevailing in the Burri slopes, while the development of the colonies took place under conditions closely simulating those in ordinary Petri dish cultures. The relationship of the figures reported in column 3 to those shown in columns 1 and 2 varies considerably from sample to sample, but in general the values resemble those in column 1 more closely than those in column 2. It is particularly noteworthy that in the cultures prepared by the modified plate method there is a complete absence of the extreme variability which characterises certain of the results obtained by the Burri technique. The mean value for the percentage errors in the modified plate method ($5{\cdot}40 \pm 1{\cdot}98$) approximates more closely to that for the plate technique ($4{\cdot}66 \pm 2{\cdot}32$) than to the figures for the Burri slope cultures ($10{\cdot}90 \pm 3{\cdot}58$). Further, the last two results are near enough to the corresponding values in Table III to justify the view that the data in Table V are practically as representative as the more extensive series. The conclusion that the conditions of growth rather than the method of application of the inoculum are mainly responsible for the erratic results observed in Burri smear-cultures appears, therefore, to be warranted.

Discussion.

The results of this investigation clearly demonstrate that more accurate determinations of the bacterial content of milk samples can be made by ordinary quantitative plating than are possible when the Burri technique is employed. Although Dorner and Demont have claimed that the smear-culture method is not less accurate than plating, it has been shown that, when the limitations proposed by these workers are applied to their results, the latter also support the conclusion reached in the present series of studies.

In an investigation of the factors mainly responsible for the inferiority of the loop-slope to the pipette-plate technique an examination has been made of the accuracy of loop measurements and of the relative effects of the conditions for growth on the surface of slopes and in Petri dish cultures upon the agreement of parallel determinations. It has been shown that when not more than two loopfuls of liquid are taken for each determination platinum loops of 1 c.mm. capacity are less accurate than pipettes, but that the inferiority of loops to pipettes can be almost entirely eliminated by employing for each determination ten loopfuls of the material to be examined. It has also been demonstrated that milk bacteria develop more erratically on slopes than when they are embedded in a solid medium as they are in ordinary Petri dish cultures. This factor is probably largely responsible for the lack of agreement between the results of parallel determinations made by Burri's method. It appears probable that on the surface of agar slope cultures many organisms experience greater difficulty in initiating growth than when they are largely or completely surrounded by the medium. It has been observed, for example,

that certain milk streptococci will grow in Petri dish cultures in which the agar has been inoculated while still liquid although they entirely fail to develop on the surface of slopes of the same medium. Further, it has been noted that erratic results observed on Burri slopes are frequently due to the growth of groups of bacterial colonies of types not represented on the other parallel cultures. A possible explanation of this phenomenon is that by the proliferation of other organisms small areas are rendered suitable for the growth of bacteria which ordinarily do not develop upon slope cultures of the medium.

Although the Burri method is not sufficiently accurate for general use in the routine examination of milk samples, it may still prove to be of value for particular purposes. For the detection of sources of contamination in the production and distribution of milk, for example, it will doubtless prove to be a useful method. In this connection the simplicity of the technique and the fact that it can be applied outside the laboratory are important points in its favour.

SUMMARY.

1. A comparison has been made of the accuracy of the ordinary plate and Burri smear-culture methods for the determination of the bacterial content of milk samples.

2. In determinations made by the smear-culture technique the percentage error has been shown to be markedly higher than in those made by plating.

3. Evidence is presented in support of the view that the inaccuracy of the Burri method (as ordinarily employed) when compared with the plate technique is partly due to the errors involved in loop measurements and partly to the inferiority of the conditions for growth on slopes to those in plate cultures.

We wish to express our thanks to Dr A. M. Smith for advice regarding the statistical part of this investigation.

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70. THE EFFECT OF COMMON SALT ON THE GROWTH OF LACTIC STREPTOCOCCI IN MILK

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(With 3 Figures.)

It is well known that the quantity of salt added to Cheddar cheese curd has an important influence on the quality and rate of ripening of the resultant cheese. The character of the cheese body is, indeed, dependent to a large extent on the rate of salting. An investigation of the salt content of cheeses of tender body, normal body, and harsh body showed that the percentage concentration of salt in the moisture in cheese of normal body in general did not exceed 5·5 per cent. (1). The following table, quoted from (1) summarises the results obtained:

Type of cheese	Average salt concentration	Range of salt concentration		
		Minimum	Maximum	Usual range
Tender body	4·42	2·75	6·35	3·65–5·25
Normal body	4·82	3·25	7·55	4·25–5·75
Harsh body	5·79	3·45	8·45	5·25–6·95

The retarding effect of excess salt on the rate of cheese ripening has also been demonstrated (2).

The influence of salt is no doubt due in part to its dehydrating effect on the cheese proteins; but its effect on the rate of cheese ripening has frequently been attributed to inhibitory action on the growth of lactic streptococci. Arising from the above results, therefore, it seemed of interest to study the effect of different concentrations of salt on the rate of acid production by "starter" cultures.

A considerable volume of work has been published dealing with the effect of inorganic salts on the growth of bacteria. In general, it has been found that sodium chloride in small proportions acts as a stimulant to growth, while in larger proportions it has definite toxic properties. So far as lactic acid bacteria are concerned, Orla-Jensen (3) has investigated the influence of certain concentrations of sodium chloride on the production of acid in a casein peptone broth containing 2 per cent. dextrose. He found that with most lactic acid bacteria there was no inhibition by 2·5 per cent. salt. With 5·5 per cent. salt there was very considerable inhibition, and with 10 per cent. salt

there was almost complete inhibition in every case. His experiments were, however, carried out on an artificial medium and not on milk; moreover, the concentrations investigated were limited to 0·5, 2·5, 5·5, 10·5 and 15·5 per cent. It appeared desirable to repeat his work, using milk as a growth medium, and to investigate intermediate concentrations of sodium chloride.

The present experimental work was carried out partly with a mixed commercial culture of lactic streptococci, and partly with a suspension in saline of a pure culture of lactic streptococci washed from the surface of yeast whey agar, according to the method of Whitehead and Cox(4). The culture (10 c.c.) was inoculated into a sample (190 c.c.) of fresh morning's milk of finest bacteriological quality, a separate inoculation being made for each sample in any one experiment. The weighed quantity of salt (A.R. sodium chloride) was added to the milk in the solid form in small proportions, with gentle shaking, before the inoculation with starter culture.

The acidity determinations were made by the ordinary method of titration of 10 c.c. with $N/10$ caustic soda using phenolphthalein as indicator. For purposes of comparison the results were all referred to 9 g. of original milk and represent, therefore, acidity calculated as per cent. lactic acid. The milk samples to which salt had been added were measured out with a 10 c.c. pipette, and the weight of this volume was taken for the first series of titrations. The measurement was carried out by means of a pipette until clotting took place, and thereafter a weighed amount equal to the weight of fresh sample delivered by a 10 c.c. pipette was taken. Titrations were carried out at suitable intervals, and the results, calculated as per cent. lactic acid, were plotted against the length of time from inoculation.

The results show some variation from experiment to experiment. There was always a stimulation of acid production by the addition of 1 per cent. salt, and frequently a stimulation with 2 per cent. salt. With 3 per cent. salt there was on one occasion a definite stimulation, and on other occasions a definite inhibition. Higher concentrations of salt gave very definite inhibitions of the growth of starter cultures as measured by the development of acid.

Figs. 1 and 2 are typical of the results obtained. From the standpoint of this paper the most important point established is that the growth of streptococci in milk is inhibited in progressively greater degrees by concentrations of salt from 3 per cent. to 6 per cent., and that total inhibition is approached by any concentration greater than 6 per cent. A concentration of 5 per cent. salt gives a definite but incomplete inhibition of growth. This concentration corresponds with the limits of concentration of salt in a cheese of good body, and suggests that the effect of salt on cheese "body" may, in part at any rate, be ascribed to the retardation in the growth of the lactic streptococci by the high salt concentration.

The inhibition of growth by 5 per cent. salt solution was as strong at 37° C. as at 30° C. (Fig. 3).

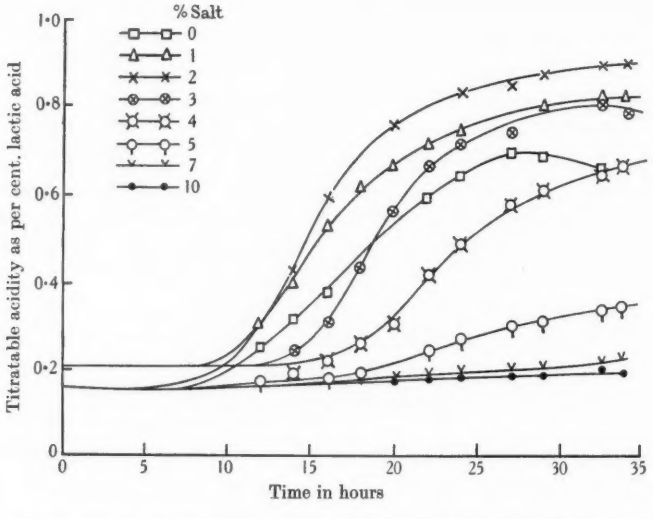

Fig. 1. Graphs showing rate of development of acid in inoculated raw milk treated with sodium chloride in different proportions.

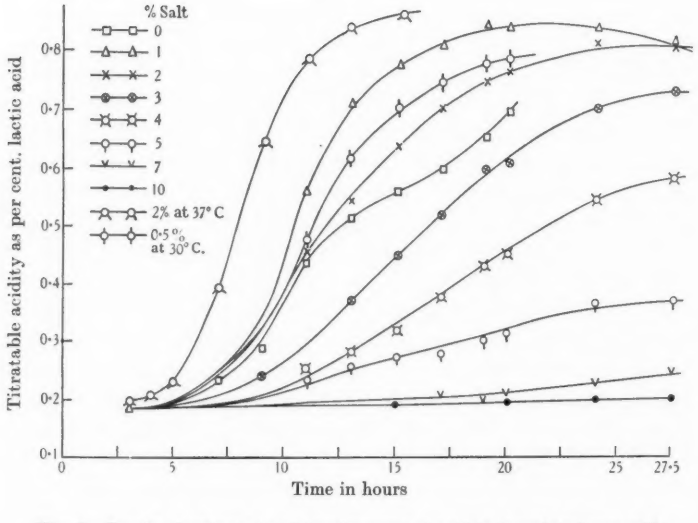

Fig. 2. Graphs showing rate of development of acid in inoculated raw milk treated with sodium chloride in different proportions.

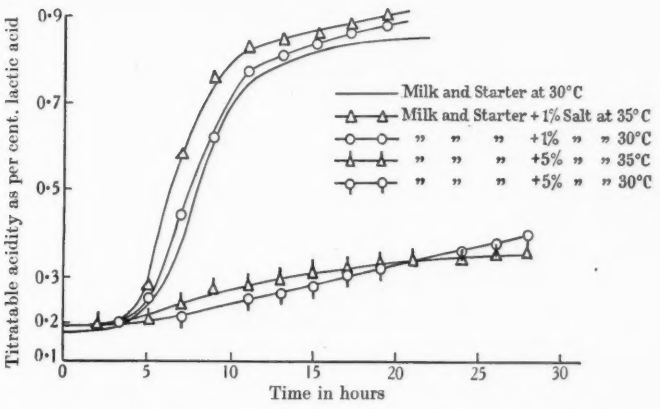

Fig. 3. Graph showing rate of development of acid at 30° C. and at 35° C. in inoculated milk treated with sodium chloride in different proportions.

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71. THE HEAT DENATURATION OF ALBUMIN AND GLOBULIN IN MILK

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(With 2 Figures.)

It has been shown by Hardy (1), Chick and Martin (2), Lepeschkin (3), Wu and Wu (4) and Lewis (5), using solutions of egg albumin and oxyhaemoglobin, that heat coagulation consists of two distinct processes: (*a*) denaturation, or the alteration of the protein under the influence of heat, which must precede (*b*) flocculation, the rapid separation of the denatured protein in the presence of a suitable electrolyte. The denaturation causes loss of solubility of the protein in water and in dilute salt solutions at its isoelectric point, and is irreversible. The rate of change is influenced by temperature, the concentration of hydrogen or hydroxyl ions, and the neutral salt content. Water is necessary for heat denaturation, but the chemical change in the protein molecules is still obscure. Wichmann (6) and Chick and Martin (2) demonstrated that no change occurs when egg albumin is heated to high temperatures in the dry state.

Denaturation and coagulation should not be confused, since coagulation includes the second process of flocculation by neutralisation of the particle charge, and denaturation may take place without being followed by flocculation. Salt-free albumin in solution will not coagulate on boiling, but will be denatured, after which precipitation will occur if a small amount of electrolyte be added. Similarly the term denaturation and not coagulation is applied to the change which takes place in the albumin and globulin when milk is heated. There is no visible coagulation, but the conditions determining the dispersion of the albumin and globulin are changed. The larger associated particles of denatured albumin and globulin are readily precipitated by acid and by salt solutions. The denaturation which takes place on heating milk is made evident, and its extent measured, by the precipitation of albumin and globulin in addition to casein when the heated milk is treated with those reagents which precipitate casein alone from unheated milk.

The heat coagulation of isolated pure proteins in solution has been studied extensively, but there is little knowledge of the effects on the lactalbumin and lactoglobulin when milk is heated, and it will readily be seen that the data available are not in agreement. Sebelien (7) observed that an aqueous solution of lactalbumin was coagulated by heating in salt solution at 72° C., while on heating milk itself Orla-Jensen and Plattner (8) found only slight coagulation

below 70–75° C. The latter authors stated that the albumin fraction could be precipitated completely after 1 hour at 77·5° C., 30 min. at 80° C., or 5 min. at 90° C. Babcock (9) found no change after 20 min. at 65° C. Freudenreich (10) reported 15–20 per cent. of the albumin and globulin to be coagulated after heating at 68–69·5° C. for 30 min. Rupp (11), heating for 30 min., found no change at 62·8° C., 5·71 per cent. coagulated at 65·6° C., 12·76 at 68·3° C., and 30·87 at 71·1° C. Weinlig (12) reported an average of 8·5 per cent. of the soluble protein to be rendered insoluble in 30 min. at 60° C., and 40 per cent. in 1 min. at 80° C. Grimmer, Kurtenacker and Berg (13), heating 1 min., found a coagulation of 5·7, 26·8, 62·0, 74·6 and 77·4 per cent. at 70, 80, 90, 95 and 100° C. respectively.

In none of these investigations has a detailed study been made of the rate of denaturation of a given sample of milk over a series of different temperatures. Moreover, the method most generally used for the determination of albumin and globulin in milk depends on the coagulation of the casein-free filtrate by heating in the presence of a little acetic acid. All the soluble protein in the filtrate cannot be recovered by this method, at the most only 70 per cent. is obtained, as heating in weakly acid solution results in the formation of non-coagulable substances. This fact explains the distribution of the nitrogenous constituents of unheated and heated milk into casein, albumin, albumose and peptone by Kieferle and Gloetzl (14).

For these reasons an investigation has been made of the amounts of lactalbumin and lactoglobulin denatured by heating portions of the same milk for varying periods at each of several temperatures. By such means the progress of the reaction could be followed at each temperature, and the effect of temperature increase observed. The proteins were separated by the reliable methods of Moir (15). The maximum amount of casein is precipitated at a *p*H of 4·5–4·7, a range which covers the isoelectric point of the protein. Heat-denatured albumin and globulin are also precipitated within this range (16), which can best be obtained in milk by a suitable acetic acid-sodium acetate buffer.

EXPERIMENTAL.

Portions of the milk were taken in $8 \times 1\frac{1}{4}$ in. glass tubes fitted with stirrers, and immersed in water, at temperatures so adjusted that all reached their respective temperatures in 75 sec. The tubes were immediately transferred to thermostats maintained at 63·0, 65·2, 67·3, 70·0, 72·4, 75·0 and 80·0° C., and after heating for periods varying from $2\frac{1}{2}$ to 60 min., removed and rapidly cooled in iced water. The water in the thermostats was covered with oil to minimise evaporation and the temperatures were constant to $\pm 0{\cdot}05$° C. The times of heating were measured to a second; evaporation from the milk was negligible.

On the unheated and heated milks determinations were made by the Kjeldahl method of (1) total protein nitrogen, (2) nitrogen of casein and

denatured albumin and globulin, and (3) nitrogen of unchanged albumin and globulin, after separating the proteins as follows:

(1) *Total protein.* 5 ml. of the milk were weighed out, 35 ml. of water added, followed by 10 ml. of 20 per cent. trichloracetic acid, giving a final concentration of 4 per cent. trichloracetic acid. The mixture was maintained at 60–65° C. for half an hour, allowed to cool, filtered, and the precipitate washed with 1 per cent. trichloracetic acid solution.

(2) *Casein and denatured albumin and globulin.* This was carried out as described fully by Moir (15), maximum precipitation at a *p*H near the isoelectric point of 4·6 being obtained by adding, to 10 ml. of milk (weighed), 50 ml. of water at a temperature of 40–42° C., 1·5 ml. of 10 per cent. acetic acid, and 4·5 ml. of *N*/4 sodium acetate as a buffer.

(3) *Soluble albumin and globulin.* Sufficient 20 per cent. trichloracetic acid was added to the filtrate from (2) to give a final concentration of 4 per cent. trichloracetic acid, and treated as in (1) above. On account of the small nitrogen contents, *N*/70 acid and alkali were satisfactorily used.

Results.

The results of heating a milk sample containing 0·3477 per cent. casein nitrogen and 0·0796 per cent. albumin and globulin nitrogen at temperatures up to 80° C. are given in Table I. The total protein content remained un-

Table I. *Unheated milk contained* 0·0796 *per cent. albumin and globulin nitrogen.*

Temp. in ° C.	Time of heating in min.	Percentage residual albumin and globulin N in heated milk	Percentage of the total albumin and globulin denatured
63·0	15	0·0726	8·8
	30	0·0702	11·8
	60	0·0680	14·6
67·3	5	0·0708	11·1
	15	0·0672	15·6
	30	0·0631	20·7
	60	0·0601	24·5
70·0	2·5	0·0691	13·2
	5	0·0663	16·7
	15	0·0621	22·0
	30	0·0571	28·3
	60	0·0505	36·6
75·0	2·5	0·0611	23·2
	5	0·0544	31·7
	15	0·0393	50·6
	30	0·0259	67·5
80·0	2·5	0·0422	47·0
	5	0·0346	56·5
	15	0·0186	76·6
	30	0·0132	83·4

changed except with the samples heated at 80° C., where there was a slight reduction. This reduction was expected, but the temperature at which it would become evident was unknown. The hydrolysis of protein disturbs the course of denaturation and prevents its true measurement; substances not

precipitable by trichloracetic acid may be produced from any of the proteins, and all the nitrogen precipitated from the acetic acid filtrate by trichloracetic acid need not be nitrogen of the undenatured albumin and globulin. Values for residual albumin and globulin nitrogen after heating at 80° C. cannot therefore be compared with those for lower temperatures. The effect of heating at the higher temperatures, resulting in an increase in non-protein nitrogen, will be investigated separately.

In a second series of experiments, the results of which are given in Table II, it was decided to omit 80° C., and to include 72·4° C., making the series of temperatures up to 75° C. more complete. The raw milk contained 0·4217 per cent. casein nitrogen and 0·0930 per cent. albumin and globulin nitrogen.

Table II. *Unheated milk contained* 0·0930 *per cent. albumin and globulin nitrogen.*

Temp. in ° C.	Time of heating in min.	Percentage residual albumin and globulin N	Percentage of the total albumin and globulin denatured
65·2	5	0·0887	4·6
	15	0·0861	7·4
	30	0·0831	10·7
	60	0·0803	13·7
67·3	5	0·0867	6·8
	15	0·0821	11·7
	30	0·0798	14·2
	60	0·0756	18·7
70·0	2·5	0·0856	8·0
	5	0·0820	11·8
	15	0·0776	16·6
	30	0·0721	22·5
	60	0·0638	31·4
72·4	2·5	0·0811	12·8
	5	0·0789	15·2
	15	0·0687	26·1
	30	0·0592	36·4
75·0	2·5	0·0759	18·4
	5	0·0700	24·7
	15	0·0558	40·0
	30	0·0443	52·4

As a guide to the variation in the extent of denaturation with different milks, the results obtained for six fresh milk samples are set out in Table III. The albumin and globulin nitrogen contents were determined before and after heating for 30 min. at 63° and 70° C.

Table III.

Milk No.	% albumin and globulin N in raw milk	% of the total albumin and globulin denatured	
		63° C.	70° C.
1	0·0796	11·8	28·3
2	0·0930	7·1	22·5
3	0·0931	10·1	27·5
4	0·0916	8·5	25·6
5	0·0924	13·5	32·3
6	0·0864	11·5	31·8

Discussion of results.

Appreciable quantities of albumin and globulin are denatured at as low a temperature as 63° C., amounting to an average of 10·4 per cent. after 30 min. heating, for the six milk samples of Table III. These are the conditions of low-temperature pasteurisation. For the same samples the average denaturation after 30 min. at 70° C. was 28·0 per cent. The series of data which has been given for two samples includes 67·5 and 52·4 per cent. of the total soluble protein denatured in 30 min. at 75° C., and an apparent denaturation of 83·4 per cent. in the one case at 80° C.

It is interesting to note that whereas the heating of normal milk causes denaturation of the albumin and globulin, but no coagulation, the heating of milks rich in soluble protein, such as colostrum, causes actual visible coagulation. In this case the proportion of globulin present is so high that the calcium caseinate present cannot protect it. During the gradual transition from colostrum to normal milk, the proportion of globulin steadily falls, and a stage is reached when there is no coagulation on heating. This would suggest that there is a minimum globulin concentration which will give heat coagulation, and from the researches of Engel and Schlag(17) on the colostrum of three cows, it would appear to be about 1 per cent., reached from 60 to 84 hours after parturition.

The relationship between temperature and time of heating.

The results of Table II are given in Fig. 1 by plotting the percentage of the total albumin and globulin denatured against the time of heating at each temperature. Smooth curves are obtained, but the rate of reaction does not follow a simple exponential law.

Calculation of the velocity constant on the assumption that the reaction is of the first order, shows the figure to become smaller as heating continues. The heat denaturation of isolated protein solutions is of the first order, although Chick and Martin(2) found that with egg albumin the rate slowed off more rapidly than the unimolecular law unless precautions were taken (addition of boric acid) to prevent change in *p*H. In the heated milk experiments the *p*H of all samples was determined by the quinhydrone electrode, and no change found. The fact that the denaturation of the protein in milk does not follow a normal course must be ascribed to the concurrent changes in state of other constituents, *e.g.* salts, of the complex system.

The figures of Table I give a similar series of curves.

The temperature coefficient.

It follows that the effect of temperature upon the reaction velocity cannot be studied by a comparison of the velocity constants at the different temperatures, as the equation to the curve is unknown. That such comparison is impossible was realised when attempts were made(18) from so-called uni-

molecular velocity constants calculated from one time of heating only at each temperature. To determine the effect of temperature it is, however, legitimate to compare from the curves the times taken at the several temperatures to bring about the same reduction in the concentration of unchanged protein. The reciprocals of these times are proportional to the average velocities of denaturation at those temperatures. The following are the available figures:

(1) 13·7 per cent. of the total albumin and globulin denatured by heating at 65·2, 67·3, 70·0, 72·4, and 75·0° C. for 60, 25·5, 8·7, 3·5, and 1·2 min. respectively.

(2) 18·7 per cent. denatured at 67·3, 70·0, 72·4, and 75·0° C. in 60, 18·6, 7·7, and 2·6 min. respectively.

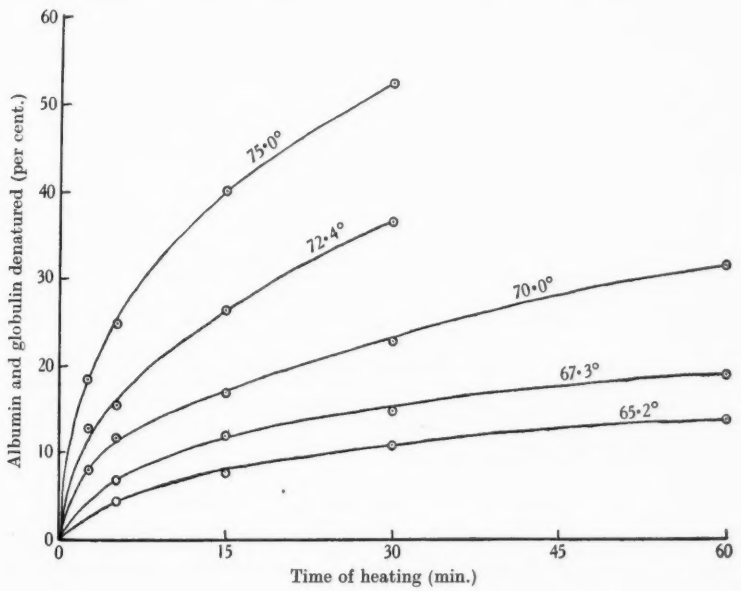

Fig. 1.

(3) 31·4 per cent. denatured at 70·0, 72·4, and 75·0° C. in 60, 21·8, and 8·2 min.

In Fig. 2 for each of these series of times, the logarithm of the time in minutes has been plotted against the temperature of heating in ° C. The points for each series fall closely to a straight line, and the three lines are parallel, indicating that the relative increase in velocity of denaturation for a rise in temperature of 1° C.—the temperature coefficient—is constant at approximately 1·5 times. This coefficient is of the same high order as those which have been obtained for the denaturation of pure protein solutions. For a temperature increase of 10° C. the same extent of denaturation would be reached 57·5 times as quickly.

The figures obtained for the other milk, given in Table I, when plotted and treated as above, show a temperature coefficient of 1·5 between 63·0 and 75·0° C. At 80° C., although the actual percentage of the protein denatured is greater than at lower temperatures, the same increase in velocity of denaturation is not maintained. This apparent check in the course of the reaction occurs at the same time as the reduction in total protein, the effect of which has already been considered.

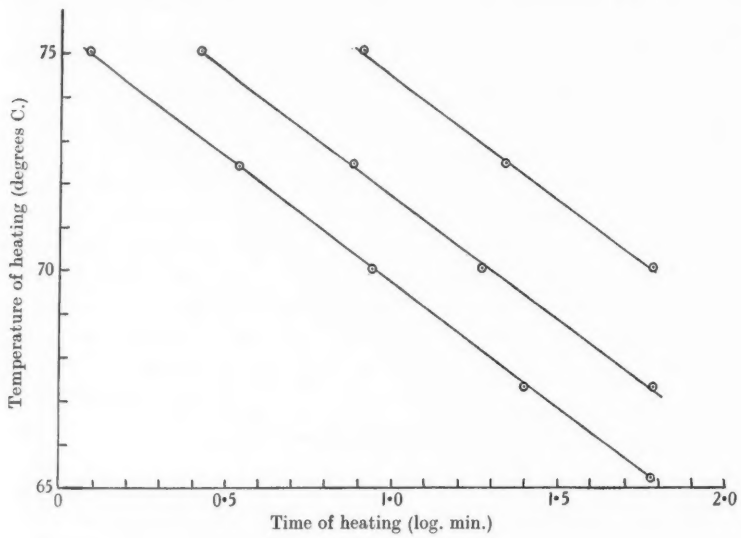

Fig. 2.

SUMMARY.

The heating of milk causes denaturation of the lactalbumin and lactoglobulin to an extent depending on both the temperature and the time of heating.

A series of data has been given for the amounts of albumin and globulin denatured on heating samples of milk at various temperatures from 63 to 80° C., for periods of from 2½ to 60 min.

An average of 10·4 per cent. of the total soluble protein was denatured after 30 min. heating at as low a temperature as 63° C.

Smooth curves were obtained for the progress of denaturation with time at each temperature, and, over the range of 63–75° C., the relative increase in velocity of denaturation for each rise in temperature of 1° C. was found to be constant, the temperature coefficient of the reaction being 1·5.

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72. PHOSPHORUS COMPOUNDS OF MILK

V. THE PHOSPHORUS PARTITION IN MILK, WITH PRELIMINARY OBSERVATIONS ON MILK PHOSPHATASE

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The importance, in the nutrition of the young animal, of the mineral constituents of milk has led from time to time to reinvestigation of the forms in which certain of these constituents occur in milk. Among these the phosphorus compounds of milk have received their share of attention. Recent work in this field has been reviewed by Allen(1), and only a few apposite references will be given in the present paper. Our knowledge in this section of the field has, however, been hampered by the lack of a fairly rapid yet reasonably accurate technique.

As is now well known, the accurate evaluation of the phosphorus partition in blood demands the destruction as early as possible after obtaining the blood sample of the enzymes which hydrolyse (and synthesise) phosphoric esters. A fairly rapid separation of the acid liquid from the precipitated colloids is also advisable if errors due to the slow hydrolysis of lipins in the precipitate by the acid are to be avoided (Kay(2)).

It seemed to us not unlikely that such considerations might apply with equal force to milk and that, with these possible sources of error in mind, a convenient and accurate method for the determination of the amounts of the various types of phosphorus compounds present in milk, based in part on methods already developed for blood, might be evolved.

The high fat content of milk introduces an initial technical difficulty. In earlier experiments by one of us(3) a method used for freeing the milk from protein and fat was precipitation with dialysed iron, which, when carried out carefully, yielded a clear filtrate containing phosphorus compounds, but neither protein nor lipin. It was found possible to show by this means that organic phosphorus compounds occur in protein-free lipin-free milk filtrates, prepared either from cow's, goat's or human milk, and that these compounds were in part hydrolysed by enzyme preparations from various mammalian tissues. But the precipitant obviously carried down large quantities of inorganic phosphate, and also undetermined, though probably small, quantities of phosphoric ester.

Lenstrup(4) developed a method which permitted, for the first time, a fairly quantitative evaluation of the phosphorus fractions in cow's milk,

though he concluded erroneously that lipins were not present to any appreciable extent. He used picric acid as a precipitant, although Lüdecke(5) had found that picric acid in the cold slowly decomposed lecithin on standing.

A very convenient method for the precipitation of proteins and fats from cow's milk has recently been indicated by Sanders(6). With the help of this technique (which consists in the addition to the milk of trichloracetic acid up to 8 per cent. strength, which completely precipitates both proteins and fats) we have made a further investigation of the phosphorus partition in cow's milk. We have paid particular regard to the acid-soluble phosphoric esters, since Sanders, using methods of phosphorus determination which appear to us to be open to objection, reached the conclusion that acid-soluble organic phosphorus is not present in appreciable quantities in such filtrates.

The methods which we have used, and which we will now briefly outline, recommend themselves on the grounds that, working with quite small quantities of milk, a large series of determinations may be carried out in a relatively short time, with an accuracy of less than ± 2 per cent. for duplicate samples. The only specialised apparatus required is a good colorimeter.

Methods.

(*a*) *Acid-soluble phosphorus.* Duplicate or triplicate 5 c.c. samples of milk are pipetted into 50 c.c. measuring flasks. 25 c.c. of distilled water are added, and 25 per cent. trichloracetic acid solution is added up to the mark. The flask is now shaken thoroughly, and allowed to stand for 10 min. The liquid is filtered through a No. 30 Whatman or suitable phosphate-free paper. *Inorganic* P is now determined on 3 c.c. duplicate samples of the filtrate, using the Briggs[1] technique(7); a 0·150 mg. standard is usually required for cow's milk.

Total acid-soluble P is determined in duplicate or triplicate by pipetting 2 c.c. samples of the filtrate into 200 mm. × 25 mm. hard glass boiling tubes[2], to each of which is added 1 c.c. of a mixture of 1 part of sulphuric and 2 parts of nitric acid by volume. The tubes are heated on a wire gauze over a micro burner (low flame) until dense white fumes appear. If the solution is not clear at this stage, a few drops of nitric acid are added, and boiling is continued until white fumes appear again. The tube is allowed to cool, two drops of superoxol or perhydrol are added, and the liquid boiled once again until white fumes again appear. The liquid should now be quite colourless. 8 c.c. of distilled water are added, the tube contents again boiled, cooled and transferred quantitatively to 25 c.c. flasks, and the Briggs procedure is again applied except that in this case 5 per cent. ammonium molybdate solution is added

[1] Denis and von Meysenbug(8) found that citric acid affected the closely related Bell-Doisy method for determination of phosphate. We found that at concentrations of citric acid (from the milk) and trichloracetic acid present in our filtrates there was no appreciable interference of these substances with the Briggs colorimetric results.

[2] We boil out any *new* tubes at least twice with the HNO_3-H_2SO_4 mixture before using them for total P determinations.

instead of the acid molybdate, the sulphuric acid being present already in the liquid. Once a little experience has been acquired, this procedure for total acid-soluble phosphorus works very smoothly. 0·150 or 0·200 mg. P standards are usually required.

(*b*) *Total phosphorus.* 2·5 c.c. of milk are accurately diluted to 25 c.c., or 5 c.c. to 50 c.c. in a suitable measuring flask, and determinations carried out in triplicate on 2 c.c. samples of the diluted milk using the procedure described above for total acid-soluble P. In some recent determinations we have found that the use of perchloric acid as an oxidising agent(9) diminishes considerably the time required for the destruction of the organic matter.

(*c*) *Lipin phosphorus.* This determination, owing to the relatively small quantity of lipin present in milk, is one of the least satisfactory of the present series. Fairly satisfactory results are obtainable as follows. 1 c.c. of milk is pipetted accurately into 35 c.c. of a mixture of 3 parts 97 per cent. alcohol and 1 part redistilled ether in a 50 c.c. flask. The contents of the flask are then boiled gently by careful immersion in hot water, cooled under the tap, and made up to the mark with the alcohol-ether mixture. Filtration is carried out with precautions to avoid evaporation, and two 20 c.c. aliquots of the filtrate are pipetted into boiling tubes as used for total acid-soluble P. The solvents are carefully boiled off, and the fat-containing residue is oxidised with the usual quantity of sulphuric-nitric acid mixture, using more nitric acid if necessary. The same terminal procedure is adopted as in the total acid-soluble P determination, except that 0·100 mg. P should be added to each of the lipin P flasks to bring their P content within the most accurately determinable range, and subtracted from the resulting determination.

Organic acid-soluble P is given by the difference between the total acid-soluble and the inorganic acid-soluble P; *total acid-insoluble* P (=casein+lipin P) is given by the difference between the total P and the total acid-soluble P. Casein P is indicated by the difference between this figure for total acid-insoluble P and that for lipin P. There is an accumulation of possible errors in the "casein P" value, and we are far from satisfied with it.

The above colorimetric procedure for total P and total acid-soluble P has been checked against a standard (gravimetric) method of analysis(10) with satisfactory results such as those given in Table I.

Table I. *Total* P *and total acid-soluble* P *in milk as determined by the colorimetric and the gravimetric* (*officially approved*) *method.*

No. of milk sample	Determination	Official gravimetric duplicates mg. per 100 c.c.		Colorimetric duplicates mg. per 100 c.c.	
1	Total P	99·7	97·7	99·5	98·8
2	,,	93·5	92·1	94·2	94·0
3	Total acid-soluble P	71·4	69·2	72·3	70·7
4	,,	77·7	78·4	78·6	78·6

Sanders(6), using the gravimetric method just referred to but for *inorganic* as well as total acid-soluble P, came to the conclusion that there was little

or no organic acid-soluble P in milk. This conclusion, which disagrees with the findings of several earlier workers (*e.g.* Hart and Andrews(11), Jordan, Hart and Patten(12), Kay(3), Lenstrup(4)), we believe to be due to the inapplicability of the method used (the method of the American Association of Agricultural Chemists for fertilisers) to the determination of inorganic phosphate *in milk*.

The results obtained by the two methods, the colorimetric and the gravimetric, are compared in Table II.

Table II. *Inorganic and total acid-soluble* P *in milk filtrates—showing inconsistency of "official" method for inorganic* P (*mg.* P *per* 100 *c.c. milk*).

	Inorganic P			Total acid-soluble P	
No. of milk sample	Official gravimetric method	Colorimetric method	Magnesia mixture method	Official gravimetric method	Colorimetric method
5	68·1	63·4	—	70·3	71·5
6	68·0	61·7	—	70·3	70·7
7	60·5	60·4	—	—	70·4
8	70·3	62·8	—	78·1	78·6
9	67·7	62·0	—	—	70·4
10	62·9	56·8	56·7	—	—
10 (duplicate)	65·0	56·4	56·0	—	—

The inorganic P as determined by the "official" method may be anything between the true inorganic P and the total acid-soluble P, depending on the extent to which the phosphoric esters in the acid-soluble fraction have been hydrolysed during the analytical procedure, which includes at one stage the heating of the strongly acid solution containing the phosphoric esters to 65° C. for 1 hour. The last two figures given in Table II indicate that the method of magnesia mixture precipitation, which does not include heating in acid solution, gives figures for the inorganic P content of the milk filtrates (trichloracetic acid filtrates after neutralisation) which agree well with the colorimetric, but not with the "official" gravimetric method. This is further evidence that the "official" procedure for inorganic P determination cannot be applied to milk filtrates, which contain labile phosphoric esters.

Quantity of acid-soluble phosphoric esters in milk.

It will have been noted that the phosphoric ester content of the milk filtrates is given by the difference of two fairly large quantities. For this reason there is a *possible* error in the phosphoric ester figures of about 20 per cent., although the error of a group of determinations will be much less than this.

In Table III are given typical results obtained with milks subjected to various treatments. It will be observed that the certified milk, obtained from superior herds, had a higher phosphoric ester content than the ordinary milk used for pasteurisation.

It must not of course be assumed that the "inorganic phosphate" as determined by this method is in fact a true measure of the amount of uncombined

phosphate present in the milk when it is withdrawn from the gland. There may be present compounds of the type of phosphagen, easily decomposed on standing for a relatively short time with strong acids such as trichloracetic or sulphuric acids at room temperature. Preliminary experiments seem to show that such compounds, if present in milk, are present to a very small extent only. Nor does the method of analysis just described distinguish between hexose phosphates, glycerophosphates and nucleotides, the last of which are almost certainly present in milk (Kay and Marshall(14)). Possible methods for distinguishing between these different types of phosphoric ester are under consideration, and the applicability to milk of methods used for their determination in blood and other tissues is being explored.

Table III. *Quantity of acid-soluble phosphoric esters present in milk.*

	Acid-soluble P content in mg. P per 100 c.c. milk			
	Free P	Total P	Ester P	Remarks
Whole milk:				
Raw milk	65·9	75·9	10·0	Ester P compounds from 1/5 to 1/10 of the inorganic P
Same milk pasteurised	67·8	75·0	7·2	
Certified milk from same dairy	71·8	85·7	13·9	
Whole milk (average of six milks):				
Raw milk	61·7	72·8	11·1	Ester P compounds from 1/4 to 1/7 of the inorganic P
Same pasteurised	62·3	72·2	9·9	
Certified milk from same dairy	63·2	80·9	17·7	
Skim milk:				
From raw milk	60·9	75·1	14·2	—
From same pasteurised	60·6	75·1	14·5	—
From certified milk from same dairy	58·5	79·3	20·8	—

Presence of a phosphatase in milk.

Early in the course of the present work it was found in a number of cases that, if the milk was kept, the inorganic phosphate content increased at the expense of the acid-soluble phosphoric ester content, whilst the other fractions remained fairly constant. This change might have been due either to bacterial action, or to direct enzymic hydrolysis of the phosphoric esters by a phosphatase secreted with the milk. It was shown by one of us in 1925 that the mammary gland contains an enzyme which will actively hydrolyse, at least in part, the acid-soluble phosphoric esters of milk, and the presence in milk of a weak hexosephosphatase was first stated by Demuth(13). Observations on the phosphatase of milk have recently been made also by Wilson and Hart(15).

We find that, even at room temperature, there is a fall in the phosphoric ester content of raw milk on standing (see Tables IV and V).

Table IV. *Effect of standing on ester* P *content of milk (mg.* P *per* 100 *c.c. milk). Holstein milk.*

	Time milk kept (hours)			
	0	4	12	24
(1) Milk cooled rapidly and kept cool	7·0	7·0	7·0	7·0
(2) Same milk cooled slowly and kept cool	7·0	7·0	6·0	6·0
(3) Same milk cooled slowly to room temperature	7·0	5·1	4·2	4·2
(4) Same milk kept in thermos flask	7·0	3·6	—	3·6

Table V. *Effect of keeping at* 38° *on ester* P *content of milk* (*mg.* P *per* 100 *c.c. milk*). *Guernsey milk.*

	Time milk kept (hours)				
	0	2	4	8	12
(1) Fresh milk	18	15·8	12·1	12·1	10·8
(2) Same milk, skimmed	—	10·7	8·4	9·3	8·6

Even in presence of chloroform, which presumably inhibits the multiplication of bacteria, the ester P diminishes on keeping the milk. In fact, in the presence of a little chloroform there may be slightly more hydrolysis than in its absence, possibly due in the latter case to the inhibitory effect of the lactic acid, produced by the bacteria multiplying in absence of chloroform, on the phosphatase, of which the optimum *p*H is decidedly on the alkaline side of neutrality (see below). In fact, the addition of dilute NaOH (2 c.c. of 1 per cent. NaOH to 100 c.c. of milk) accelerates the disappearance of the phosphoric ester, whilst the addition of dilute lactic acid inhibits this disappearance.

To demonstrate more clearly the presence of the enzyme in milk, small quantities of the fluid were added under more carefully controlled conditions to excess of Na β-glycerophosphate solutions in presence of antiseptics, and the liberation of inorganic phosphate, which takes place slowly in the milk alone by the autolysis of its own phosphoric esters, was found to be much increased by the addition to the milk of this phosphoric ester. An experiment is shown in Table VI; the milk enzyme was rather below the average activity, but the effects of addition of glycerophosphate and of pasteurisation are shown quite well. The *p*H was the same in each flask.

Table VI. *Hydrolysis of glycerophosphate by the milk enzyme* (*mixed, raw milk*).

Flask No.	Contents	mg. inorganic P per 100 c.c. of reaction mixture (hours)		
		0	20	72
1	30 c.c. 0·5 % glycerophosphate + 10 c.c. raw milk	13·8	15·5	18·1
2	30 c.c. 0·5 % glycerophosphate + 10 c.c. pasteurised milk*	13·7	13·8	13·9
3	30 c.c. 0·5 % glycerophosphate + 10 c.c. water	0	0	0
4	30 c.c. water + 10 c.c. raw milk	13·8	14·3	14·9
5	30 c.c. water + 10 c.c. pasteurised milk*	13·9	13·8	13·9

* Same milk pasteurised in the laboratory, 30 min. at 62·5° C.

The pasteurisation process appears to destroy the enzyme.

Experiments on similar lines, but in which the hydrolysis is carried out in strongly buffered solutions at varying *p*H values from 8·2 to 10·5, yield figures indicating an optimum activity in the region of *p*H 8·9 for the glycerophosphatase activity of fresh milk. A typical example is the following:

*p*H of reaction mixture	8·2	8·5	8·9	9·25	9·65	10·0
Amount of hydrolysis (mg. P)	4·8	6·9	8·3	6·6	5·7	1·7

This activity-*p*H relationship, which has been confirmed several times, indicates that milk phosphatase belongs to the group of animal phosphatases with an optimum *p*H in the neighbourhood of 9.

In the experiments shown in Table VII the phosphatase content of the milk is given by the number of milligrams of P liberated when 1 c.c. of milk is incubated for 24 hours with a mixture of 24 c.c. of glycine buffer at *p*H 8·9 and 25 c.c. of 0·5 per cent. Na β-glycerophosphate at *p*H 8·9. The value is of course purely arbitrary and the figures shown in Table VII are not numerically comparable with those obtained by the standard technique, to be described in a later paper, for the determination of the phosphatase of milk.

Phosphoric ester and phosphatase content of milk as affected by the breed of cow.

The evening milk of 18 cows, 6 Holstein-Friesians, 6 Ayrshires and 6 Jerseys, none either at the beginning or the end of the lactation period, has been analysed by the methods just described, and in addition, phosphatase estimations and fat determinations have been made in each case.

The collected results are given in Table VII.

Table VII. *Phosphorus partition in, and phosphatase and fat content of, the evening milk of* 18 *cows of three different breeds.*

P given as mg. P per 100 c.c. milk, phosphatase in arbitrary units (explained in text), fat in percentage.

		Acid-soluble P			Acid-insoluble P		Phos-phatase	Fat
Cow No.	Total P	Total	Inorganic	Ester	Total	Lipin	units	%
Holstein-Friesians:								
3	87·4	69·2	61·6	7·6	18·2	4·0	11·5	3·6
4	83·9	64·5	56·8	7·7	19·4	4·3	9·5	3·6
7	104·7	81·2	73·5	7·7	23·5	5·1	21·5	4·1
12	90·7	73·0	65·3	7·7	17·7	4·3	9·9	—
24	96·3	64·5	57·4	7·1	31·8	4·3	8·6	3·3
31	95·9	73·9	63·2	10·7	22·3	6·0	7·8	3·6
Average	93·1	70·9	62·9	8·0	22·1	4·7		
Ayrshires:								
41	112·3	83·6	76·5	7·1	28·7	4·1	17·4	5·0
43	126·5	97·0	88·0	9·0	29·5	4·3	18·3	5·2
46	112·6	81·3	72·7	8·6	31·3	4·6	12·6	4·9
55	93·4	69·6	58·0	11·6	23·8	5·5	6·6	4·5
56	94·7	73·0	61·7	11·3	26·7	5·6	6·3	4·4
58	92·9	66·9	54·6	12·3	26·0	5·2	6·3	4·0
Average	106·2	78·5	68·6	9·9	27·6	4·9		
Jerseys:								
64	108·6	84·0	63·9	20·1	24·6	12·9	—	—
65	118·6	86·5	76·8	9·7	32·1	13·5	19·9	6·2
66	126·0	91·2	81·0	10·2	34·8	8·7	14·5	6·2
68	112·8	86·1	73·7	12·4	26·7	9·7	10·6	5·7
70	97·9	74·9	62·3	12·6	23·0	8·5	5·9	4·7
77	107·8	82·0	70·0	12·0	25·8	6·7	13·2	5·9
Average	111·9	84·1	71·3	12·8	27·8	10·0		
Average for all	103·7	77·8	67·6	10·2	25·8	6·5		

An influence of the breed on the phosphorus content of the milk seems likely from this table. In total phosphorus per 100 c.c. milk, in total acid-soluble phosphorus and in ester phosphorus there is an increase from the Holstein-Friesians through the Ayrshires to the Jerseys. There is also the

suggestion of a correlation between the total fat and the lipin P, and between the phosphoric ester P and the phosphatase content. The total P of the milk and the phosphatase content seem to vary together, *whereas in general the ester* P *appears to vary inversely as the phosphatase.*

This last correlation is to be expected, if, as seems not unlikely from our findings, the phosphoric ester content of the milk is diminishing from the time it is drawn (and possibly from the moment it is secreted) until the time when the proteins are precipitated. If other conditions are the same, the amount of phosphoric ester surviving hydrolysis in this interval will be greater or less as the enzyme content of the milk is small or large. It is thus possible that under natural conditions the calf might receive in its mother's milk, sucked from the gland at frequent intervals, a considerably greater amount of phosphoric ester than would be expected on the basis of the analysis of the fluid removed from the udder twice only per day. This possibility is being further investigated.

Summary and conclusions.

1. Using the method described by Sanders for protein and fat precipitation from cow's milk, a fairly satisfactory method of determining the phosphorus partition in cow's milk has been devised.

2. It is found that the gravimetric procedure of the American Association of Official Agricultural Chemists ((10), p. 3), which was not, in any case, specifically recommended for the analysis of milk, cannot be applied with safety to the determination of inorganic phosphate in filtrates prepared by Sanders' (1932) method, and that the conclusion that there is no appreciable amount of organically combined phosphorus in these filtrates is therefore invalidated.

3. The ester P (organically combined, acid-soluble P) in milk obtained by the usual milking routine varies from 1/7 to 1/4 of the inorganic P of the milk (from 7 to 21 mg. P per 100 c.c. milk).

4. An active phosphatase is present in cow's milk, and brings about changes in the ester P content of the milk on standing, possibly within and certainly without the mammary gland. Like phosphatases of all mammalian tissues, its optimum *p*H is in the neighbourhood of 9·0. In freshly drawn milk it is working, therefore, considerably on the acid side of its optimum.

5. The phosphoric ester P of the milk appears to vary with the breed of cow, being low in the milk of Canadian Holstein-Friesian cows and higher in that of Jersey cows.

We are much indebted to Dr E. W. Hammond of the Toronto City Dairy Co., who supplied us with many of the commercial samples of milk used in our experiments.

One of us (W. R. G.), wishes to acknowledge his indebtedness to the Government of the province of Ontario whose Fellowship in Agriculture was held during the progress of this work.

NOTE ADDED JULY 3, 1933.

Since the foregoing work was submitted for publication, three papers dealing with acid-soluble organic phosphorus compounds in milk have come to hand (Lang and Miethke(16), Hochheimer(17) and Bomskov(18)). All agree that such compounds are present in cow's milk to a greater or lesser extent; human milk and goat's milk are also reported to contain such esters (confirming the previous findings of one of us(3)). Endeavours have been made by all of these workers to partition the phosphoric esters into fractions, on the lines used by Lohmann for the partition of the organic phosphorus compounds in muscle, but in this case the findings of the three workers are by no means in agreement. We hope to return to this question in a later paper.

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73. PHOSPHORUS COMPOUNDS OF MILK

VI. THE EFFECT OF HEAT ON MILK PHOSPHATASE. A SIMPLE METHOD FOR DISTINGUISHING RAW FROM PASTEURISED MILK, RAW FROM PASTEURISED CREAM, AND BUTTER MADE FROM RAW CREAM FROM THAT MADE FROM PASTEURISED CREAM[1]

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(With 2 Figures.)

In a previous paper(1) we described some of the properties of the phosphatase present in raw milk, and stated that pasteurisation of milk appears to destroy this enzyme. In the present paper will be described (*a*) a quantitative method for the determination of the amount of this enzyme in a given sample of milk, (*b*) the effect of heat on the rate of destruction of this enzyme, and (*c*) a simple qualitative method capable of being used in an ordinary milk-testing laboratory for differentiating between raw and pasteurised milk and milk products.

A. Quantitative determination of phosphatase in milk.

The method is founded on that previously described by one of us(2) for the determination of phosphatase in blood plasma or serum. The quantity of the enzyme present in cow's milk is, however, considerably greater than that present in human (or bovine) blood plasma, so that in the former case both the quantity of the enzyme-containing fluid used and the time of action on the substrate may be considerably diminished. On the other hand, since phosphoric esters capable of hydrolysis by the enzyme are present in milk in considerably greater amounts than in blood plasma, it is necessary, for quantitative determinations in milk, to carry out additional control and blank experiments. Nevertheless, by working in glycine buffer solutions at a *p*H (8·9) near to that of the optimum *p*H for the milk enzyme, the experimental technique may be simplified.

Solutions required.

(1) *Glycine buffer.* 7·505 g. pure glycine ("amino-acetic acid A.R.") and 5·85 g. pure sodium chloride are dissolved together in 1 litre of distilled water. A few drops of pure chloroform may be added as a preservative. 320 ml. of the

[1] A short summary of conclusions reached in the earlier stages of this work was communicated to the Royal Society of Canada (Section V) on May 19, 1933, at the Kingston meeting.

solution are mixed with 80 ml. of $N/10$ NaOH. This gives a buffer solution of *p*H 8·9 at 37° C., which is best kept tightly stoppered in the refrigerator.

(2) *Sodium glycerophosphate solution.* 1·5 g. of crystalline Na β-glycerophosphate[1] are dissolved in 1 litre of water, and the solution adjusted to approximately *p*H 8·9 with a little *N* NaOH, using phenolphthalein as indicator and the glycine buffer solution (1) as the standard buffer for comparison.

(3) *Magnesium sulphate solution.* 1·2 g. $MgSO_4.7H_2O$ are dissolved in 100 ml. water.

(4) *Phosphate reagents* (Briggs). (*a*) *Acid molybdate.* 10 g. of pure ammonium molybdate A.R. are dissolved in 100 ml. of warm water, and the solution added slowly to a cooled mixture of 30 ml. strong H_2SO_4 and 50 ml. water. The whole after cooling is made up to 200 ml. (*b*) *Sodium sulphite.* A 20 per cent. solution in water of pure crystalline sodium sulphite is made up. (*c*) *Hydroquinone solution.* A 0·5 per cent. aqueous solution of hydroquinone, acidified by the addition of 1 ml. of 10 per cent. sulphuric acid to each 100 ml. is used.

(5) *Trichloracetic acid solution.* A 25 per cent. aqueous solution of the A.R. acid is used.

(6) *Standard phosphate solution.* 0·4390 g. pure dry KH_2PO_4 is dissolved in water and made up to 1 litre. Each ml. contains 0·1 mg. P.

Method. The sample of milk, which should be dealt with without undue delay (preferably within 24 hours of sampling, and after keeping as near ice temperature as possible) is diluted with two parts of distilled water to one of milk. Eight test-tubes (each large enough to hold 20 ml. ($6 \times \frac{5}{8}$ in.) and provided with a well-fitting rubber stopper) are filled as follows:

A_1 and A_2. 5 ml. glycine buffer, 5 ml. glycerophosphate solution, 1 drop magnesium sulphate, 2 drops chloroform.

B_1 and B_2. As A_1 and A_2.

C_1 and C_2. 5 ml. glycine buffer, 5 ml. distilled water, 1 drop magnesium sulphate, 2 drops chloroform.

D_1 and D_2. As C_1 and C_2.

To each tube is added 0·5 ml. of the diluted milk. 5 ml. of 25 per cent. trichloracetic acid are immediately added to tubes B_1, B_2, D_1 and D_2, in order to stop enzymic activity and precipitate the milk proteins. These tubes are stoppered and shaken, and placed at once in the refrigerator.

The other four tubes, A_1, A_2, C_1, C_2, are placed in a water thermostat at 38° C., and the time noted. At the end of 3 hours, they are removed, cooled to room temperature by plunging into cold water, and 5 ml. of trichloracetic acid are added to each tube. They are re-stoppered and shaken, and allowed to stand at room temperature for 15 min. In the meanwhile the tubes B_1 and B_2, D_1 and D_2 are removed from the refrigerator. After 15 min., the contents of the eight tubes are filtered through 10 cm. Whatman No. 30 filter

[1] Obtainable from Boot's Research Chemicals Department, Nottingham.

papers (phosphate-free), into eight clean test-tubes. After filtration, 10 ml. of liquid are pipetted from each test-tube into a 25 ml. measuring flask. Similar measuring flasks containing 0·5, 1 and 2 ml. of the standard P solution are prepared, 3 ml. trichloracetic acid and a little water added to each.

To all the measuring flasks are now added 2 ml. of each of the three phosphate reagents (*a*), (*b*) and (*c*), the liquid is made up to the mark, and the contents of the flasks thoroughly mixed. After standing for 25 min., the blue colours which develop are compared with the nearest standard in a colorimeter, and the free phosphate present in the 25 ml. flasks is thus determined.

Calculation. The difference between the mean of C_1 and C_2 and the mean of D_1 and D_2 gives the "autolysis P" which is usually very small. The difference between the mean of A_1 and A_2 and that of B_1 and B_2 gives the "enzyme+autolysis P" figure. The "autolysis P," if any, is now subtracted from the latter and the result multiplied by $\frac{15{\cdot}5}{10} \times \frac{1}{0{\cdot}5} \times \frac{3}{1} = 9{\cdot}3$ to give the amount of phosphatase (in arbitrary units) present in 1 ml. of the original milk. A unit of phosphatase (milk) is taken as the amount of enzyme which at 38° C. in glycine-NaOH buffer at *p*H 8·9, and in presence of slight excess of Mg ions, will liberate inorganic phosphate equivalent to 1 mg. P from excess of Na *β*-glycerophosphate in 3 hours.

B. Effect of temperature on the phosphatase content of milk.

Using the method just described, or slight modifications of it, for phosphatase determination, the stability of milk phosphatase at various temperatures has been investigated.

The technique employed for heating the milk at the lower temperatures, when the destruction of enzyme is slow, is quite simple. At the higher temperatures, where the destruction of the enzyme is rapid, difficulties arise as a result of the lag in the heating and cooling of the milk, which cannot be overcome entirely by diminishing the quantity of milk taken or by increasing the area of the heating surface.

The results of our experiments, of which a fairly large number have been done at different temperatures, are summarised in Table I and are shown

Table I. *Rate of destruction of milk phosphatase at various temperatures.*

Temperature (° C.)	Percentage of original phosphatase destroyed in											
	0·33	1	2	3	5	10	15	20	25	30	60	120 min.
75	96·2	.	98	.	98	.	.	.	.	.	.	.
70	.	88	.	98	98	.	.	.	.	.	.	.
67·5	.	77	89	.	97	100	.	.	.	.	.	.
64·5	.	.	.	.	88	99	98	98	.	.	.	.
62·5	.	.	.	.	86	94	98	98	98	.	.	.
60	.	.	.	.	61	.	85	.	.	94	98	.
55	.	.	.	.	.	.	37	.	.	56	73	.
50	.	.	.	.	.	.	.	.	.	.	19	38

graphically in Fig. 1. It will be seen that at temperatures of 75° C. or above the enzyme is almost instantaneously destroyed. At temperatures in the "holder" pasteurising range, the rate of destruction is smaller, but 96 per cent. destruction or above is readily obtainable in 15–20 min. at 62–63° C. This finding has been repeated in many experiments, and there is no doubt that the ordinary methods of "holder" pasteurisation either in the laboratory or on the commercial scale result in the practically complete destruction of the milk phosphatase.

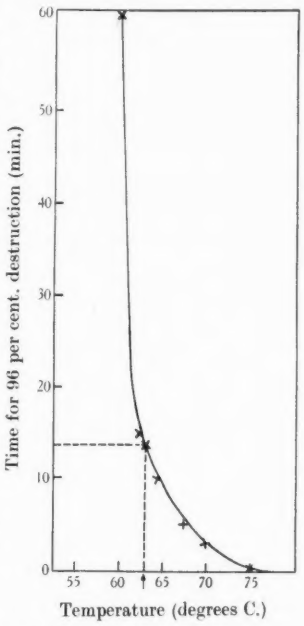

Fig. 1. Relationship between temperature and time required for 96 per cent. destruction of milk phosphatase. Dotted line shows the relationship at the lowest temperature for official pasteurisation (62·8° C.).

Milk pasteurised in the laboratory.

For milk pasteurised under proper conditions in the laboratory at 62–63° C. for ½ hour, typical figures showing the destruction of the enzyme are given in Table II. Each of these experiments was done under slightly different conditions with regard to the phosphatase content of the original raw milk, the volume of milk used, and the time taken by the milk to reach the pasteurisation temperature. All, however, indicate equally that "holder" pasteurisation for 30 min. if properly carried out results in at least 98 per cent. destruction of the phosphatase originally present in the milk. In fact the first 15–20 min. at 62–63° C. is sufficient to destroy the enzyme almost completely. Exps. 1 and 2 were on milk from individual cows of known high phosphatase content, Exps. 3 and 4 were on the mixed milk of eight or more cows.

Table II. *Effect, on the phosphatase content of whole milk, of "holder" pasteurisation in the laboratory.*

	Phosphatase content of the milk, in arbitrary units per ml. (units defined on p. 65)			
	Exp. 1*	Exp. 2†	Exp. 3*	Exp. 4‡
Before heating	1·39	2·65	0·48	0·44
Heated 5 min.	0·79	.	.	0·34
,, 10 ,,	0·38	1·34	0·07	0·16
,, 15 ,,	0·06	0·58	0·03	0·02
,, 20 ,,	0·01	0·27	0·01	0·00
,, 25 ,,	0·00	0·09	0·01	0·02
,, 30 ,,	0·02	0·05	−0·01	0·01
,, 35 ,,	.	0·02	0·01	.
,, 40 ,,	.	0·00	.	.

* Temperature raised to 62·5° C. almost at once. † Temperature raised to 62·5° C. in 10 min.
‡ Temperature raised to 63° C. in 8 min.

Milk pasteurised commercially.

Samples of commercially pasteurised milk were obtained from various reputable distributing firms, in Toronto, in Reading, and in London, and their phosphatase content determined. In Table III twelve figures are given for such samples, which may be compared with a similar number of figures for raw milk, and for milk pasteurised in the laboratory. It is considered that those commercial samples marked with an asterisk in Table III are almost certainly improperly pasteurised.

Table III. *Phosphatase content of commercially pasteurised milk, of laboratory pasteurised milk, and of raw, mixed milk, in arbitrary units per ml. milk.*

Pasteurised		
Commercially. Units phosphatase	In laboratory. Units phosphatase	Raw. Units phosphatase
0·02	0·01	0·87
0·00	0·01	0·76
−0·01	0·02	0·68
0·02	−0·01	1·17
0·07*	0·02	0·89
0·09*	0·00	0·85
−0·02	0·01	0·54
0·03	0·03	0·85
0·06*	0·00	0·81
0·04	−0·02	0·57
0·04†	−0·03	0·53
0·00	0·01	1·05
Averages, excluding negative and asterisked figures:		
0·021	0·012	0·797

† Pasteurised and homogenised.

N.B. Figures in the same horizontal line do *not* indicate the same milk, pasteurised and raw. All the 36 figures shown are from different samples of milk having no relationship to each other.

The pasteurised milks shown in Table III were in each case the product of responsible well-known distributors. In Table IV are given the designation, the (quantitative) phosphatase content, and also the results of the qualitative test (to be described in section C below) for a number of somewhat doubtful samples of milk from various producers and middlemen, which were recently submitted to a public health laboratory for bacteriological report.

It will be observed from Table IV that out of the thirteen alleged raw samples to be reported on, at least four and probably five had been heated, whereas out of the three samples alleged to be pasteurised one had certainly not been properly heated. To the bacteriologist such information with regard to the samples submitted to him for report is obviously important.

Effect on phosphatase of adding raw to pasteurised milk.

Samples of mixed raw milk, of a fairly low original phosphatase value, were mixed with various quantities of the same milk properly pasteurised, in order to find out what percentage addition of raw milk to pasteurised could be detected by the phosphatase technique.

The results of such an experiment are shown in Table V. Even with a milk originally rather low in phosphatase content, an addition of 5 per cent. of raw milk can be determined with certainty. Gould's(3) test, based on milk amylase, seems to be rather more sensitive than this.

Table IV. *Phosphatase content of certain doubtful samples of "raw" and "pasteurised" milk.*

Sample No.	Milk reputed to be	*Qualitative test. Phosphatase present or absent	Quantitative test. Units of phosphatase present	Conclusions. Milk
48	Raw	+	0·91	Raw
49	Pasteurised	+	0·57	Raw or very poorly pasteurised
50	Raw	+	0·38	,,
51	,,	+	1·16	Raw
52	,,	+	1·07	,,
53	,,	+	0·58	,,
54	,,	+	0·68	,,
55	Pasteurised	−	0·00	Pasteurised
56	,,	−	0·01	,,
57	Raw	+	1·43	Raw
77	,,	+	0·14	Poorly pasteurised
78	,,	−	0·00	Pasteurised
79	,,	−	0·00	,,
80	,,	+	1·05	Raw
84	,,	−	0·00	Pasteurised
85	,,	−	0·04	,,

* See section C.

Table V. *Effect, on the phosphatase content of the mixture, of the addition of small quantities of raw to pasteurised milk.*

Mixture		
Pasteurised parts	Raw parts	Phosphatase units
100	0	−0·02
99	1	−0·02
97	3	0·03
95	5	0·056
90	10	0·074
85	15	0·10
75	25	0·13
50	50	0·23
0	100	0·57

Rate of disappearance of phosphatase compared with rate of bacterial mortality on pasteurisation.

Assuming that *B. tuberculosis* is the most thermostable of the pathogenic organisms that may be present in milk, it is of interest to compare the thermal death-rate of these organisms with the rate of destruction of phosphatase by heat. Such a comparison may be made by using our own phosphatase destruction figures, and the death-rate for the tubercle bacillus given by North and Park(4). If, as suggested by Dahlberg(5), the *logarithm* of the time for complete destruction of the tubercle bacillus is plotted against the temperature, a straight line results. If on the same diagram is plotted the logarithm of the

time for 96 per cent. destruction of phosphatase, the points also fall on a fairly straight line. A comparison of the position of the two lines will show whether or not the destruction of phosphatase in milk can serve as an index of the destruction of *B. tuberculosis*, and therefore of the other less stable pathogenic organisms.

It will be seen that at any temperature from 60 to 75° C. (140–167° F.) 96 per cent. destruction of phosphatase indicates complete destruction of *B. tuberculosis*.

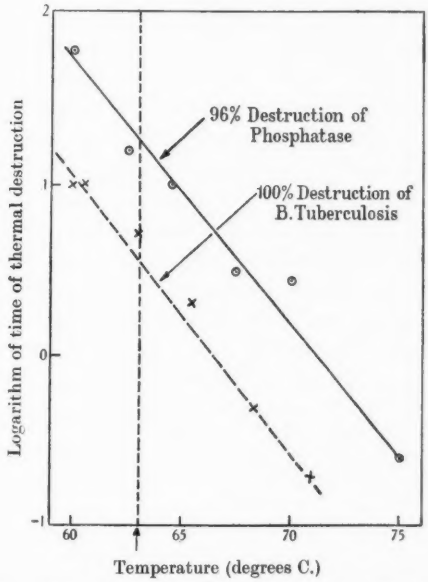

Fig. 2. Showing that complete destruction of *B. tuberculosis* in milk occurs somewhat before 96 per cent. destruction of phosphatase. Continuous line shows relationship between log. of time required for 96 per cent. destruction of phosphatase in milk and the temperature. Dotted sloping line shows relationship between log. of time for complete destruction of *B. tuberculosis* in milk and the temperature. Dotted vertical line shows minimum temperature for official pasteurisation.

In two instances the rate of destruction of phosphatase and that of the mixed organisms of milk were compared by direct determination, at intervals during pasteurisation, of phosphatase and of bacterial population by plate count, using the same sample of milk. One of these experiments is given in Table VI.

It may, then, be concluded that absence of phosphatase from a heated milk provides evidence of the practically complete destruction of the normal bacterial flora, and, in particular, indicates that any living tubercle bacilli will have been completely destroyed by the heating process. It naturally tells us nothing regarding the organisms which may have got into the milk *after* pasteurisation.

Table VI. *Rate of destruction of phosphatase and of bacteria in milk by heat ("holder" pasteurisation).*

Time of heating min.	sec.	Time at 62·5–63° C. to nearest min.	Phosphatase units	Living bacteria per c.c.	*B. coli* in 1/1000
		0	1·66	58,400	+
*3	35	0	1·00	110	–
8	15	5	0·28	18	–
13	00	9	0·14	6	–
18	08	15	0·05	1	.
23	00	19	0·03	1	.
28	15	25	–0·01	1	.
33	00	29	–0·01	2	.

* For the milk to reach 62·5° C. required 3 min. 35 sec.

Is the enzyme associated with the milk fat?

It was noticed early in the course of the work on milk phosphatase that the enzyme appeared to be associated to a considerable extent with the milk fat. Thus the enzyme content of whole milk was always higher than that of skimmed milk, or of the fat-free layer obtained after centrifuging, although on reconstituting the whole milk as nearly as possible, the total enzyme content was not greatly altered. The enzyme was actually concentrated to a considerable extent in the fatty layer. If, however, the cream was pasteurised, its phosphatase content was completely or almost completely destroyed.

This suggested to us the possibility that the phosphatase test might also be used for determining whether a given sample of butter had been made from fresh or from pasteurised cream.

On further investigation it was shown that the phosphatase test could, in fact, be used in this way. Two examples of the use of this method are given in Table VII. In each case a quantity of raw cream was divided into two

Table VII. *Effect of pasteurisation of cream on the phosphatase content of butter.*

	Phosphatase content per c.c. (in the case of butter, per g.)	
	Exp. 1	Exp. 2
Whole milk	0·40	0·94
Skim milk	0·31	0·59
Raw cream	0·82	1·93
Butter from raw cream	0·27	0·46
Buttermilk from raw cream	0·71*	1·78
Pasteurised cream	0·01	0·01
Butter from pasteurised cream	0·00	0·03
Buttermilk from pasteurised cream	0·01	0·01

Expt. 1: milk poor in phosphatase. Expt. 2: milk rich in phosphatase.

* This figure is only approximate.

halves, one half was pasteurised, and the other not. Butter was made from each half of the cream. Phosphatase determinations were made on the original milk, cream and skimmed milk, and on the butter and buttermilk.

The method of determining the phosphatase in butter is not entirely satisfactory, but gives results which have definitely more than qualitative signifi-

cance. A weighed quantity of butter (1·0–1·5 g.) is shaken up with exactly 25 ml. of glycine buffer at *p*H 8·9 (solution 1, section A) previously warmed to 37° C. Of the emulsion formed, 5·5 ml. are taken, run into 5 ml. of glycerophosphate, and the rest of the procedure is essentially as in section A, except that control tubes C_1, C_2 and D_1, D_2 are unnecessary. Another method which will probably be found of greater value in deciding whether a butter has been made from raw or from pasteurised cream is to melt the butter in a 50 ml. centrifuge tube at about 40° C. and centrifuge. The aqueous layer separating is found to have an astonishingly high phosphatase content if the butter has been made from raw cream, but very low or zero content if the butter has been made from pasteurised cream. In the former case it is, indeed, necessary to dilute the aqueous layer ten to twenty times before its enzyme content can be conveniently determined by the routine method.

The foregoing results also indicate that the phosphatase is not directly associated with the fat itself, but is present either in the very thin, mainly protein layer which covers the fat globules or is adsorbed on the fat globules in such a way that the greater part of it may be removed in the buttermilk by changing the fat-in-water emulsion (cream) to one of water-in-fat (butter). The further fact that, on centrifuging the melted butter, nearly all the enzyme present is found dissolved in the aqueous layer shows that the enzyme is probably not even in part fat-soluble.

C. QUALITATIVE METHOD FOR DISTINGUISHING BETWEEN RAW AND PASTEURISED MILK, OR BETWEEN BUTTERS MADE FROM RAW AND PASTEURISED CREAM.

This method is merely a qualitative modification of the quantitative method described in section A. The same solutions are required, but filtration and quantitative colorimetry are avoided. It may, however, be claimed for the method that with reasonable care, and using a simple technique, it is possible to decide fairly quickly whether a given sample of milk or cream is raw or pasteurised.

Method. The sample of milk or cream, which should be dealt with preferably within 48 hours of sampling, is divided into two portions, *A* and *B*. *A* is diluted forthwith with 2 parts of distilled water to 1 part of milk or cream, while *B* is first raised to the boil, or the test-tube containing it is held in boiling water for 3 min. (taking care that none of this sample escapes the heating) and then quickly cooled and diluted similarly. Four 20 ml. test-tubes, fitted with good rubber stoppers are then labelled A_1, A_2, B_1, B_2. Into each tube is pipetted 5 ml. of glycine buffer (solution 1, section A) and 5 ml. of glycerophosphate solution (solution 2), 1 drop of magnesium sulphate solution (solution 3) and 2 drops of chloroform are then added to each tube.

To each of the tubes A_1 and A_2 0·5 ml. of the diluted milk *A* is added, and to each of B_1 and B_2 0·5 ml. of the diluted (but previously heated) milk *B*.

The four tubes are now stoppered, inverted once or twice, and placed in a water bath at 38° C. (or in a beaker of water raised to 38° C. and then placed in an incubator at the same temperature).

Tubes A_1 and B_1 are removed after 3 hours, and cooled to room temperature. To each tube is now added 2 ml. of each of the acid molybdate, sulphite, and hydroquinone solutions (4 (*a*), 4 (*b*), and 4 (*c*)). the contents mixed, and the tubes placed in a test-tube rack for 15 or 20 min. After this time, the blue colours produced are compared either in a rack or a suitable comparator block. The precipitate formed does not seriously interfere with this comparison. If tube *A* is darker in colour than tube *B*, then the original milk or cream is either unpasteurised or improperly pasteurised. If the two colours are the same, the original milk has been heated, and that to a sufficient extent to destroy harmful organisms. In case of doubt, tubes A_2 and B_2 are allowed to remain at 38° C. for another 3 hours or more, and again tested. If pasteurisation has been effectively performed, there should still be no difference in colour between A_2 and B_2.

Application to butter.

This method, with the modifications described on p. 71 can also be applied to butter. Approximately 1 g. of butter is emulsified in 25 ml. of warm glycine buffer, and 5 ml. of the emulsion used instead of 5 ml. of glycine + 0·5 ml. of diluted milk. Alternatively the aqueous layer, which separates after melting at 40° C. and centrifuging the butter, may be used, preferably after dilution.

D. General discussion.

If some constant constituent of raw milk were found, of which the rate of destruction by heat was just sufficiently great to bring about its complete disappearance from English milk by an exposure of the milk to a temperature of $147{\cdot}5 \pm 2{\cdot}5°$ F. for 30 min. (or from Canadian or American milk by an exposure to a temperature of $142{\cdot}5 \pm 2{\cdot}5°$ F. for the same period of time), but which was not appreciably diminished by shorter exposure at these temperatures or by longer exposures at lower temperatures, an ideal method could be devised for deciding in the laboratory whether or not correct "holder" pasteurisation of a given sample of milk had taken place. Such a milk constituent still remains to be discovered. A number of milk constituents, having a greater or lesser thermal stability, have, however, already been recognised, and several of these have been recommended by different workers, as convenient test substances for enabling a decision to be made as to whether or not a sample of milk has been heated.

Of these substances some, such as the peroxidase of the Storch reaction and the Schardinger enzyme, are somewhat too thermostable to use for the satisfactory detection of "holder" pasteurisation. Others, such as amylase, are a little too thermolabile, this enzyme being destroyed (Orla-Jensen (6))

by heating for ½ hour at 52·5° C. Others again require serological reagents which are not always readily available.

The agglutinin of Orla-Jensen (6, 7), which controls the rate of rise of cream particles in milk, and the extent of destruction of which has been employed by him as a very useful method for determining the type of heating to which a milk has been exposed, appears to have intermediate thermostability, being a little more thermostable than phosphatase. A fairly accurate estimate of the thermal history of a given sample of milk might of course be obtained in a sufficiently well-equipped laboratory by using several of these methods at once on the same sample of milk.

While phosphatase falls a little short of the ideal milk constituent in the sense just mentioned, the phosphatase technique we have described for detecting and controlling the efficiency of pasteurisation possesses certain very definite advantages. Thus (1) phosphatase is present in every sample of raw milk examined; (2) it is completely destroyed by efficient pasteurisation, either by the "holder" or the "flash" method; (3) it is only relatively slowly destroyed by temperatures below 60° C., so that milk which has been "pasteurised" for ½ hour at a temperature below 60° C. still shows the presence of the enzyme; (4) milk phosphatase is easily recognised qualitatively and easily estimated quantitatively; (5) either a simple qualitative test or a rather less simple, but still easy, quantitative method is available; (6) most pathogenic bacteria are destroyed by heat more rapidly than the enzyme is inactivated; (7) similar methods using the presence or absence of the same enzyme as a criterion of pasteurisation may be applied to cream or butter; (8) the total expenditure of time and laboratory materials required to determine the enzyme quantitatively is relatively small; (9) the enzyme itself, as it occurs in milk under ordinary conditions, is fairly stable, and, particularly if the milk be kept cold, suffers little change in quantity for days.

SUMMARY.

A method is described for the quantitative determination of phosphatase in raw milk. Using this method, it has been found that phosphatase is sufficiently thermolabile to be destroyed completely by pasteurisation if this process is properly carried out. The absence of phosphatase from a sample of milk or cream indicates with a fair degree of certainty that the milk or cream has been heated sufficiently to destroy such pathogenic organisms as were originally present, though it is of course no guarantee that the product is free from these organisms at the time of testing.

A simple, qualitative test-tube method is described which may be used for differentiating between raw and heated milk, or between raw and heated cream, and which with slight modifications may also be used for distinguishing between butters made from raw or from heated cream.

One of us (W. R. G.), wishes to acknowledge his indebtedness to the

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION C. DAIRY CHEMISTRY.

THE progress of dairy chemistry during the last two years will be reviewed under the following broad subdivisions of the subject:

I, Composition of milk; II, Chemistry of the constituents; III, Physical chemistry; IV, Chemistry and technology of milk products; V, Processing; and VI, The analysis of milk and its products.

Some aspects of the variation in the composition of milk have already been reviewed (Mackintosh (1)), and a fuller discussion will follow in a later review on "The Physiology of Dairy Cattle." The chemistry of the vitamins will also be dealt with in a later review on "The Nutritional Value of Milk."

I. THE COMPOSITION OF MILK.

(*a*) THE AVERAGE COMPOSITION OF MILK.

The accumulation of information on the average composition of milk is presented in Table I.

Table I. *Published data on the average proximate composition of milk.*

Investigators	Fat %	Solids-not-fat %	No. of samples	Country
Golding *et al.*(2)	3·88	8·91	312	England (South)
Golding *et al.*(2)	3·89	8·89	3,115	England (South)
Baker and Cranfield(3)	3·61	8·91	*ca.* 300,000	England (Midlands)
Szanyi(4) (1927)	3·64	8·86	4,148	Hungary
Szanyi(4) (1929)	3·77	8·73	1,304	Hungary
Szanyi(5)	3·45	9·25	739	Hungary

It can be seen that the average value for solids-not-fat is almost identical with that found by Richmond. Greater variation is observed in the average fat content. The last three lines in Table I refer to Hungarian cows, the first two for all breeds in the years cited, and the last for Allgäuer cows only.

The cases of adulteration of milk reported by the Ministry of Health (6) annually account for roughly 9 per cent. of the samples examined by public analysts. The figures are:

1929–30: 361 out of 4206 samples (8·6 per cent.);
1930–31: 399 out of 4390 samples (9·1 per cent.);
1931–32: 421 out of 4604 samples (9·1 per cent.).

Golding *et al.* (2), commenting on the analyses of 1074 samples of morning milk, state that 13·69 per cent. of the samples have fallen below 3·0 per cent. n fat content; all the evening samples have contained over 3·0 per cent. fat. Only four samples fell below 8·5 per cent. in solids-not-fat content in the morning milk, while 26 samples of the evening milk fell below standard.

(*b*) Composition of the milk of other species.

The following compositions and ranges of compositions of milk of mammals other than the cow have been reported:

Species	Total solids	Fat	Protein	Lactose	Ash	Density
Vixen (7)	18·13	6·30	6·25	4·56	0·96	—
Yak (8)	—	4·23–8·50 (6·09)	—	—	—	—
American buffalo (9)	12·97–13·67	1·83–1·69	4·20–4·88	3·73–5·74	0·86–0·96	1·042
Mare (10)	5·93–18·74 (10·96)	0·09–7·88 (1·59)	0·55–7·60 (2·69)	1·65–8·78 (6·14)	0·28–0·95 (0·51)	—

(Average values are given in brackets.)

Linton (10) finds that there is no marked difference in the composition of the milk of English and Continental mares except in the heavier breeds where the ash averages 0·51 per cent. for British and 0·38 per cent. for Continental breeds. Mares' colostrum is the same as that of cows. A deficiency of lactose is harmful to the foal. Aged mares were found to secrete richer milk.

II. THE CHEMISTRY OF MILK CONSTITUENTS.

(*a*) Milk-fat.

Considerable work has been done on the effect of the fat fed in the ration on the composition of milk fat and the consistency of butter, while the glyceride structure of butterfat has been extensively studied by Hilditch and his associates. Much attention has been paid to the auto-oxidation of butterfat and fats in general.

Buschmann (11) states that the feeding of large quantities of fat to cows alters the physical and chemical properties of milk fat to an extent comparable with the butterfat containing 18 per cent. of the fat fed. Hansson and Olofsson (12) have observed that fodder composition and the method of butter preparation affect the consistency of butter. Grass, clover and coarse green fodder give a soft fat of high iodine value (> 40). The feeding of silage and coarse winter fodder neutralises this effect and gives a hard solid butter of low unsaturation. Roots and cabbage have the same effect as straw, but clover hay raises the iodine value slightly above that from timothy hay. Of home-grown concentrates, barley gives a hard butter and legume seeds a hard brittle butter. The oil content of the cereals has a marked effect, oats and mixed grain giving a softer butter than barley. The effect of purchased concentrates depends on their fat content and the iodine value of the fat. The best consistency is given by an oil-cake containing 5–6 per cent. of oil of an iodine value of 100–110 (earth-nut, cottonseed, soya and sunflower *meal*, crushed barley, mixed grain and wheat bran). Sunflower, sesame, linseed, soya bean and rape-seed *cakes* give soft butter, while palmnut, coconut, babassu and oil-cake meals of low fat content give hard butter. The reaction of the cow to the fodder occurs in 7–8 days, but individual cows will differ in response by from 5–7 units in iodine value. Maize oil (13), fed at the rate of 1 lb. daily, although not causing any effect on fat percentage or total fat production, causes an increase of 30 per cent. in the iodine value of the butterfat and a decrease in the amount of the volatile fatty acids to half the normal value. The feeding of sugar-beets increases the Reichert-Meissl value and lowers the refractivity of butterfat (14). Hansen and Steensberg (15) set the iodine values of soft butterfat above 35, of butterfat of satisfactory consistency at 29·4–34·5, and of hard brittle butterfat below 29. They have found that feeding palmnut and coconut cake gives a colourless butter. Hilditch and Sleightholme (16), when comparing the butterfat from cows fed on coconut fat (relatively saturated)

and soya-bean oil (relatively unsaturated) as cakes, have found that, in the case of the former, the fully saturated fraction increases in butyric and lauric acid content, while the unsaturated fraction is normal; in the case of soya-bean oil, on the other hand, the fully saturated components show little change, but the lower acids in the unsaturated fraction are increased. The unsaturation of the fat in the food thus exerts a twofold influence on butter consistency in lowering the melting point of the unsaturated glycerides by increasing both their unsaturated and their lower fatty acid contents.

The study of seasonal changes in the composition of butterfat has shown that the influence of the fat in the diet is very small when compared with other causes. Thus, there are profound changes in the fatty acid composition brought about by a change from indoor to outdoor life associated with the general change in diet and temperature (17). The high iodine value of autumn butterfat decreases steadily during the indoor winter feeding period to a minimum. This is followed by a sharp rise in the spring when the animals are turned out to grass.

When the Reichert-Meissl value falls below 24 the addition of foreign fat to butter is suspected. Arup (18), in his studies of the composition of Irish winter butter, has found that a considerable number of samples fall below this minimum value, and that various factors associated with winter feeding and management conditions are conducive to the yielding of butterfat low in content of volatile fatty acids. Continental experience in this direction shows the reverse to be the case; Hungarian cows have been found to give the maximum Reichert-Meissl values in winter and minimum values in summer (19). The variation in Reichert-Meissl values has ranged from 22·6 to 31·7 (average 26·94), and the Polenske value from 1·8 to 5·8. The xylene and Kirschner values are raised in a parallel manner to the Reichert-Meissl values.

Previous methods of determining the detailed composition of oils and fats had one great drawback in that quantities of oleic esters appeared in all the fractions during the fractional distillation of the alkyl esters of the component fatty acids. This has been overcome by the acetone-permanganate oxidation method of Hilditch (20) in which the unsaturated *glycerides* are quantitatively separated as *acids* by the process of regulated oxidation of the carbon atoms, associated with the double bonds, to carboxyl groups, the fully saturated esters, isolated in the same process, yielding clear-cut fractions in the subsequent distillation of the methyl esters of the component fatty acids. Hilditch has applied this method extensively to the study of the variations in butterfat composition cited above and of the general fatty acid distribution in the mixed glycerides of butterfat.

When synthetic mixed glycerides are prepared from various combinations of the higher saturated acids with unsaturated acids (85 per cent. oleic, 15 per cent. linoleic acids), the curve connecting the molar percentage of fully saturated glycerides with the molar percentage of saturated acids is almost identical with that relation found in a number of natural animal fats including butter, tallow and some vegetable seed fats. The fully saturated glycerides do not appear in appreciable amount until the molar percentage of the saturated fatty acids exceeds 60 per cent. The vegetable fats show a tendency to an even distribution of the fatty acids (21). The component acids of the fully saturated fraction of "normal" butterfat are present in approximately the same quantitative amounts, and the composition of the fully saturated glycerides tends to be the same irrespective of the unsaturation of the fat. The combined butyric-lauric acid, and the myristo-palmitic acid contents of the whole fats and of their two divisions, fully and non-fully saturated glycerides, show similar concordance. The content of unsaturated acids in the non-fully saturated glycerides increases steadily with increase in unsaturation of the fat. The addition of a relatively saturated fat to the diet does not affect the non-fully saturated glycerides,

whereas a predominating unsaturated fat does, although the composition of the saturated glycerides is not affected. It thus appears that the study of the glyceride structure of butterfat affords more evidence of the effect of the food fat than only the determination of the amounts of fatty acids in the fats.

Lea's method (22), later modified by Taffel and Revis (23), of determining the progress of autoxidation of a fat by determining the peroxide content constitutes a great advance in the means of studying fat deterioration. Taffel and Revis have used the method to distinguish between oils "blown" at low and high temperatures since, in the former case, the peroxides are more labile and oxidise hydriodic acid more readily. Briggs (24), during investigations on the autoxidation of butterfat, has found that the acid, iodine and peroxide values remain practically unchanged during the induction period, that the peroxide value does not agree with the oxygen absorbed, that the iodine value decreases more nearly in proportion to the oxygen absorbed and that the formation of acid is a secondary process. The Fellenberg test is shown early in the induction period and does not increase whilst the Kreis test appears later and increases in intensity slowly during the latter part of the induction period and rises sharply during the period of rapid oxygen absorption. Metallic catalysts exert a hastening effect in the decreasing order: sodium vanadate, copper, iron, nickel; zinc has a slightly retarding action. Hydrogen peroxide, ultra-violet light and fat peroxides have a strong pro-oxidative effect. Lactic acid shows a moderate pro-oxidative effect. Humidity, glycerol, triolein, lactose, iodine and potassium iodide have no effect, whilst curd has a slight inhibiting action on autoxidation. With reference to the formation of the lower fatty acids during autoxidation, Elsdon *et al.* (25) have found that the Reichert-Meissl values of various oils which have developed tallowiness show an increase, while the Polenske values rise slightly, this giving the impression, in the case of margarine, of a higher butterfat content than is actually the case. With butterfat the Reichert-Meissl values rise very slightly with the development of rancidity.

The effect of sunlight in catalysing fat oxidation in milk with the associated development of a reducing potential which can be detected by methylene blue or potentiometrically has been shown by Whitehead (26) to be capable of interfering with the ordinary methylene blue test for milk. The unsaturated esters of milk fat are responsible, although Aikins and Fay (27) state that stearate also causes a rapid fall of potential. Fat is claimed to heighten the zone of reduction of methylene blue, thus causing a more rapid bleaching as compared with bacterial endoenzymes. The oxidised flavour of milk fat has been further studied by Weich and Bauer (28) (emery taint) and Kende (29), the latter stating that an enzyme, *oleinase*, may cause it indirectly. Tracy (30) finds that traces of copper, exposure to sunlight and storage at low temperature (4° C.) encourage the development of a "cappy" flavour in milk. Traces of copper and iron in frozen cream give a metallic taste and an oxidised flavour (31).

Hilditch and his associates (32) have found that the induction periods of autoxidation for oils are longer than that of the fatty acids and that the period of rapid oxygen absorption occurs almost instantaneously after treating the oil with sodium hydroxide or boiling dilute hydrochloric acid. They conclude that certain antioxygenic substances are present, varying with the oil. Further, the antioxygenic material can be removed on boiling with water but the addition of quinol causes the fat to recover its oxygen resistance. Mattill (33) finds that *o*- and *p*-dihydroxy-phenols are active antioxidants but that the naturally occurring sterols are inactive and has demonstrated the presence of pro- and antioxygenic factors in the unsaponifiable residue of the fats. Carotene (34) has been proved to be a naturally occurring antioxidant whilst the lipase of unpasteurised sweet cream butter may be considered also to possess the same property (35). Kende (29) states that there are certain antioxidants

derived from the food which resist the development of an oxidised flavour in milk, green food being rich in such factors.

With respect to food-packing material and the effect of sunlight, Coe and Le Clerc (36) have found that the exclusion of light retards the development of tallowiness and that the greater portion of the visible spectrum is inactive, especially the band 4900–5600 Å (green light). They suggest the use of black or green containers as packages for foods subject to autoxidation.

Taufel and Thaler (37) have proposed a colour test for the methyl ketones formed in the development of ketonic rancidity of fats in which the steam distillate is shaken with purified salicylaldehyde and a deep rose or blue colour is given on the addition of concentrated sulphuric acid.

The usual constants do not serve to distinguish colostrum fat from normal milk fat (38). Laxa (39) has investigated the fat of sow's milk and finds that at ordinary temperatures it is granular and of a light yellow-brown colour. The content of volatile fatty acids and capric acid is low (Reichert-Meissl, 2·1; Polenske, 1·2). The iodine value is high (58·2).

(*b*) Lactose.

The form of lactose which crystallises from aqueous solution is the α-monohydrate. Hockett and Hudson (40) report the crystallisation of the anhydrous α-form from solution for the first time. The monohydrate is shaken with methyl alcohol containing 5 per cent. hydrochloric acid, when a change to a needle type crystal (5 α-lactose 3 β-lactose) with initial $[\alpha]_D^{20}$ 67·9°, and final 55·2°, occurs. A diamond-shaped α-lactose monohydrate has been isolated from sandy ice-cream (41). This form, usually not found in ice-cream, is the type found in sweetened condensed milk. The high viscosity of the milk causes the slow growth of large lactose crystals. The use of corn sugar (glucose) instead of cane sugar for ice-cream manufacture has been suggested (42). Whitnah (43), using washed yeast for the selective fermentation of dextrose and measuring the rotatory power of solutions before and after fermentation, states that from 0 to 0·35 per cent. of glucose occurs in normal milk. The amount of glucose is not dependent on that of other constituents.

Polonovski and Lespagnol (44) claim that there are two sugars other than lactose present in human milk. These are described: *gynolactose*, M.P. 205° C.; $[\alpha]_D$ −27°; *allolactose*, M.P. 165° C.; $[\alpha]_D$ +20°. The experimental evidence of their presence has been deduced from the divergency found between their optical rotation and their reducing powers. They also report the occurrence of a laevo-rotatory glucoprotein, a lactoside, in human milk, which yields a reducing substance on hydrolysis (45).

Kometiani (46) confirms the fact that heat causes the formation of lactic and formic acids from lactose at temperatures between 80 and 120° C., thus producing a slight increase in the acidity of boiled milk. The inverse isotonic relationship of lactose and chlorides in milk has been further confirmed (47, 48, 49).

(*c*) Proteins.

Improvements in the determination of milk proteins have been devised by Moir (50), as a result of which future investigators should be able to interpret their data on a sounder analytical basis. There is reason to believe that the separation of albumin and globulin is not satisfactorily accomplished by Moir's method in the presence of artificially added blood serum proteins (51), and it is probable that a more dilute magnesium sulphate solution is necessary for casein *plus* globulin precipitation. Sanders (52) advocates the precipitation of milk proteins in an 8 per cent. solution of trichloracetic acid.

The variation in protein distribution with solids-not-fat content has been investi-

gated by Davies (53), who finds that in *normal* milk the nitrogen distribution is as follows: protein nitrogen, 94 per cent. (comprising casein, 76 per cent., albumin, 12 per cent., globulin, 6 per cent.); non-protein nitrogen, 6 per cent. As the solids-not-fat decrease, less nitrogen occurs as casein and more as globulin and (to a smaller extent) as albumin. With very low solids-not-fat, the albumin and globulin nitrogens are high and variable in consecutive samplings. Milk of low solids-not-fat content takes on the composition of a true milk fraction diluted with a liquid rich in blood constituents.

The chemistry of casein has been pursued further in the direction of its non-homogeneity and molecular weight and of its degradation by different reagents. Carpenter and Hucker (54) have fractionated casein by the acid-alcohol method and have obtained three fractions of molecular weight 98,000, 188,000 and 375,000. They were able to differentiate between these fractions by serological methods and to prove that the alcohol-soluble protein of Osborne and Wakeman was not identical with the last fraction. Demanez (55) has confirmed their work and has also shown that the caseins of the milk from the cow, sheep and goat are identical while those of mare's and human milk are different. Carpenter (56) has found the molecular weight of crude casein, determined by the Svedberg ultracentrifugal method, to be 98,000. The serological differentiation of casein and para-casein has been accomplished (57). Sasaki (58), using serological methods, has concluded that casein is the only heat-stable protein in milk, and that the formation of antibodies is most rapid with casein and slowest with albumin. He has found milk albumin and serum albumin to be different. Peskett and Folley (51) has found varying amounts of serum albumin in milk by serological methods. Cherbuliez and Schneider (59) have separated casein into fractions differing in sulphur and phosphorus content by means of 5 per cent. ammonium chloride solution. They describe fully the various properties of the fractions. Iodised casein (60) and desaminocasein (61), particularly the doubled cystine content of the latter, have been further investigated. Masui (62) has found that the nitrogen distribution of methylene casein is identical with that of true casein but that their solubility properties differ. A nitro-casein having unique colloidal properties has been described (63).

The decomposition of casein by heating in non-aqueous solvents has been investigated with the object of separating unchanged polypeptide fractions of the casein molecule. Heating in resorcinol (64) gives acropeptides (16 amino acids) comparable to those obtained by heating in glycerol (65) or by pancreatic digestion. The use of sulphuric acid instead of hydrochloric acid and prolonged autoclaving has yielded cyclopeptides as products of hydrolysis (66), while the disintegration of casein in molten acetamide (200° C.) forms closed-chain products of low molecular weight, which are hydrolysable by alkali (67). The mechanism of hydrolysis of casein by acids and alkalies has been studied (68). The digestion of casein by pure intestinal juice (69) occurs only very slowly but the process follows the lines of normal proteolysis. Arginase sets free 50 per cent. of the arginine of casein in 3 hours as against 45 per cent. liberated by trypsin (70).

Bergren (71) has found the phosphorus content of acid-precipitated casein to be 0·805 per cent. She states, however, that casein of lower phosphorus content (0·29 per cent.) can be prepared by the dialysis of milk for 36 days. The phosphorus is more loosely bound than is usually thought. Fürth and Minnibeck (72) have found 5·7 per cent. proline and 2·1 per cent. of oxyproline in casein while Pirie (73) has isolated 1 per cent. of menthionine, precipitable as its mercury salt, from casein hydrolysates.

The existence of alcohol-soluble proteins in cheese and peptic digests of casein has been proved by Allen (74). The proteins differ from casein in composition and have a low phosphorus content.

The refractivity of alkali-caseinate solutions varies with the alkali and the casein concentration owing to different degrees of association, swelling, and hydration (75), except at an alkali concentration of 0·05 *N*, at which the refractivity is independent of casein concentration. The influence of the temperature of drying of casein on the borax solubility test shows that heating to 55° C. has no effect on the solubility whilst heating up to 120° makes it harder to dissolve and gives a viscous solution. Heating above 120° causes the casein to swell but not to dissolve (76). There is no correlation between optical activity and adhesive strength.

A study of the absorption spectra of alkali solutions of casein shows that the change due to enolisation of the peptide linkages is only discernible at high temperatures or at the end of racemisation. Two types of peptide linkages have been found, those changing slowly to the enol form, which is rapidly converted to the keto form, and those yielding the stable enol form (77). The insufficiency of the keto-enol transformation theory of Dakin to explain racemisation has been pointed out by Csonka (78) since casein digests after 52 days are still optically active. The absorptive powers of casein solutions for lead and for copper (79) have been shown. That the latter quantitatively displaces potassium from caseinate proves that the copper is not joined to NH_2 groups.

Beau (80) advances as a theory of rennin precipitation that coagulation occurs by polymerisation, or by the physico-chemical condensation of casein with the phosphocaseinate of calcium. CaO and P_2O_5 act as *plastifiers* in the reaction. Svedberg (81) is of the opinion that molecular association occurs during the precipitation of general proteins like albumin and globulin and that in their natural state they occur as molecules of low molecular weight, association taking place during the processes of isolation which involve precipitation.

The similarity of the non-protein, or *residual* nitrogen of milk to that of blood or urine has been pointed out. Kieferle (82) gives a detailed analysis of this fraction. The dialysable nitrogen of milk contains a substance giving a strong ninhydrin reaction (83). Boiling milk and lactic acid fermentation increase, while coagulation decreases, the amount of dialysable nitrogen. Very little free ammonia is detected in the dialysate.

(*d*) Enzymes.

Progress in the study of the enzyme content of milk has consisted of the isolation of the peroxidase, the differentiation between xanthine-dehydrogenase and the Schardinger enzyme, and advanced studies of milk catalase and amylase. Zaykorskii and Aleksejeff (84) have published a useful titrimetric method of estimating milk catalase by determining the amount of hydrogen peroxide broken up in 30 min. at room temperature. Roeder (85) has found that the enzyme is not absorbed at the fat globule interface like the dehydrogenases and that its activity is lowered by conditions of acidity. The catalase content of milk is related to that of the blood while milk fat is another important factor determining the amount (86). That catalase is a native enzyme of milk has been confirmed by Burstein and Frum (87) and Reid (88). Reid finds that it appears in milk 7 days before calving, increases in the colostrum and remains high for 14 days. Heating gradually inactivates it, a temperature of 90–92° C. for 20–30 min. being required for complete inactivation.

Schenk (89) states that the colostrum-free milk of the cow, horse, cat, dog and guinea pig contains only traces of amylase but that the amount in human milk is high. Goat secretions contain no amylase. Schlack (90) confirms this but finds mare's milk high in amylase. Gould (91) and Orla-Jensen (92) have applied tests depending on the destruction of amylase at 60° C. for the detection either of insufficiently pasteurised milk or of the addition of raw to pasteurised milk.

The similarity in action of traces of heavy metals and of the peroxidase of milk has been demonstrated (93) but the colour given by metals in the Storch reaction is reddish-grey and brown in alkaline solution, whereas the peroxidase gives a blue colour, turning to red with alkali. Arakawa (94) describes a sensitive reagent for the peroxidase of human milk and states that the milk of rabbits on a vitamin B-deficient diet yields no peroxidase tests, although the blood peroxidase is strong. Grimmer and Engel (95) have found that digestion with pancreatin increases the peroxidase reaction and that the enzyme is closely related to albumin and is probably in loose combination with it. Although Grimmer and Engel have failed to isolate the enzyme, Elliot (96) has been able to prepare it from milk by fractional precipitation with ammonium sulphate and to examine its action on different substrates.

Wieland and his associates (97, 98) have found that the dehydrogenases of milk are adsorbed on the fat globule surface. The separation of the xanthine-dehydrogenase can be effected by adsorption on calcium chloride leaving the aldehyde-dehydrogenase in solution. The latter is responsible for the dismutation of aliphatic and aromatic aldehydes and for the oxidations in the presence of methylene blue. Wieland and Mitchell (98) by a further series of oxidation reactions have confirmed the individualities of these two enzymes.

The inactivation of lipase in butter by traces of heavy metals has been studied (35). Peptidases are not native to milk but are of bacterial origin. The rennin produced by *B. prodigiosus* is highly thermostable and acts on heated or even autoclaved milk (99).

(*e*) Mineral matter.

Allen has written a survey of the knowledge of the mineral constituents and citric acid content of milk (100).

Porcher and Brigando (101) reiterate the opinion that calcium and phosphorus are present in milk both in the crystalloid and colloid form. The dialysate of milk on evaporation yields tricalcium phosphate which is not present as such in the original dialysate. The casein complex (casein + $CaHPO_4$ + $Ca_3(PO_4)_2$) can be separated completely by porcelain filtration and can be coagulated with salt-free rennet only when dispersed in the serum and not in distilled water unless some calcium ions are present. The ultra-filtrable calcium in human milk is twice that of cow's milk but the ultra-filtrable phosphorus is lower. Acidity increases the ultra-filtrable calcium and phosphorus. With rennin coagulation, 60–70 per cent. of the phosphorus and 45 per cent. of the calcium are found in the whey, but the amounts of these constituents in the ultra-filtrable form are the same as in the original milk. Peptic and tryptic digestion increase the amount of these constituents (102). Mai (103) states that cow's and goat's milk contain 33 mg. and 23·4 mg. per cent. respectively of the acid-soluble organic phosphorus as lactacidogen. The percentages of the total phosphorus in ester combination with sugar are: cow 4·4, goat 3·07, human 10·3. Milk also probably contains a creatine-phosphorus compound and mammary tissue contains phosphoric esters. Sanders (104) uses a final concentration of 8 per cent. trichloracetic acid for precipitating milk proteins. All the calcium, magnesium and acid-soluble phosphorus under such conditions appear in the filtrate. From 68·4 to 81·9 per cent. of the total phosphorus is acid-soluble and milk high in acid-insoluble phosphorus is high in casein.

Schneck and Görgel (105) have further studied the alkali ratio (K_2O/Na_2O) of milk and have found it to vary from 0·3 to 1·7, considerable fluctuations in Na_2O content being experienced. The CaO content under these conditions is fairly constant. Steffen (106), on the basis of the alkali ratio, classifies secretions into two classes: those similar to lymph (colostrum, urine, bile) and those similar to cell contents

(milk, saliva, gastric and pancreatic juices). The potassium content of colostrum is low.

Wurster (107) finds no connection between the calcium content of the soil and the calcium content of the milk produced on it or the coagulability of the milk with rennet.

The pentabromacetone method for determining citric acid has been modified by Kometiani (108). The oxidation of citric acid by acid permanganate in the presence of bromide yields pentabromacetone which reacts with sodium iodide in alcoholic solution liberating six equivalents of iodine per molecule of citric acid. Citric acid is not detectable in human milk until 3 days *post partum* after which it occurs to the extent of 0·035–0·125 per cent. (109).

(*f*) Minor constituents.

Nakanishi (110) reports the following *cholesterol* contents in the milk of various species (mg. per cent.): sow 145, rabbit 109, cat 63, bitch 55, ewe 22·5, human 16·4, goat 14, cow 12·7, mare 11·5. The amount present is in rough proportion to the fat content.

Sasaki (111) states that the lecithin of milk is a mixture of myristo-lauro and lauro-palmito lecithin, milk fat containing 0·09 per cent. of the former and 0·19 per cent. of the latter. About 18–20 mg. per cent. of lecithin-phosphorus occurs in milk fat. Little seasonal variation occurs (112).

The incidence of traces of heavy metals in milk has been further studied. Sato (113) finds the following *manganese* contents of milk: cow's colostrum, 0·16–0·06; cow's milk, 0·02–0·04; mare's milk, 0·03–0·04; ewe's milk, 0·05–0·09 parts per million. Peterson (114) finds 0·028 p.p.m. Richards (115) reports 0·04–0·05 p.p.m. with little geographical variation. The copper content of milk has been variously reported: 0·15–0·20 (116), 0·15–0·65 (117), 0·5 (cream) (118); human milk, 0·5–0·6 p.p.m. (116). The iron content of milk has been found to be: cow's milk, 1·1–4·5 p.p.m.; human, 2·9–4·5 (119), 1·5–2·4 (117) p.p.m.

The increase in iron and copper content of milk by processing and in the iron and copper contents of dairy by-products have been studied (117, 118). A copper content of 1·5 p.p.m. is sufficient to cause the development of an oxidised flavour in milk of good quality in cold storage within 24 hours. The adsorption of heavy metal proteinates to the fat globule surface with a consequent higher ratio of metal to curd nitrogen in butter than in cream or milk has been demonstrated (120).

Sato (121), investigating the zinc content of milk, finds 7·35 (3 days *post partum*), 1·23 (25th day) and 3·89 p.p.m. (36th day) in human milk; 13·6 (colostrum), 2·34–2·12 (2nd–3rd month), 4·58 (end of lactation) averaging 3·3 p.p.m. in cow's milk; and 2·45–2·90 p.p.m. in ewe's milk.

The ease with which iodine enters milk fat from foods containing varying quantities of iodine makes it impossible to make a geographical survey of the iodine content of natural food. The maximum iodine content is in colostrum, after which the amount falls to a minimum. Thyroidless goats produce milk of low iodine content (122).

The passage of nicotine into human milk after excessive smoking has been demonstrated (123) in a case where the poisoning of the infant was suspected.

III. PHYSICAL CHEMISTRY OF MILK.

(*a*) Physical equilibria.

Mohr and Brockmann (124) have found that the stalagmometric method gives higher results than the static ring-torsion balance method for determining the surface tension of milk. The results are, nevertheless, parallel with a difference of roughly 8 dynes per cm. on an average of 45 for the static method. The surface tension falls

with increasing fat content because of the concentration of the capillary active substance at the fat globule interface and the amount of the depression varies with the state of the fat, whether solid or liquid. Pasteurisation, sterilisation, boiling, ageing, freezing and the addition of formaldehyde have practically no effect on the surface tension. Surface tension is decreased by souring but is increased by increasing the amount of air incorporated into the milk. Structural form and film phenomena account for anomalies with skim milk.

The foaming problem has also been studied by the same investigators (125). They find that milk with the greatest surface tension and viscosity gives the most stable foam and that the addition of sodium citrate greatly increases the solidity and stability of the foam. The tendency to foam depends on the tendency to form a stabilised surface. Surface viscosity is related to the ability to foam since the viscosity depends on the solidification of protein in the films. An optimum surface viscosity is required to give a stable foam. Foam formation and stability decrease with increased fat content; the amount of foam is greater when the fat is liquid than when solid. Homogenisation decreases foaming in skim milk, but increases foaming in whole milk. Repeated foaming does not, while churning does, reduce foam volume. Temperature effects are variable, but there is an optimum from 30–60° C.

The factors influencing the viscosity of cream have been further investigated by Babcock (126). Pasteurisation lowers and homogenisation increases viscosity whilst the ageing of pasteurised cream increases the viscosity though not to the same extent as in raw cream. Cream which has been both pasteurised and homogenised, does not increase in viscosity with ageing. Freezing lowers the viscosity which is not restored on ageing. Acidity has but a slight effect unless over 0·3 per cent. Raw or pasteurised cream cooled slowly has a higher viscosity than that with rapid cooling. The lower the separation temperature, the higher is the viscosity. Viscosity is influenced by length of time and temperature of storage.

Marquardt and Dahlberg (127), investigating the creaming of milk pasteurised at high temperatures, have found that milk exhibits a heat induction period before the creaming properties are affected. This varies considerably for different samples of milk. To influence creaming ability the relation of the time of exposure (Y) to the degree Fahrenheit of heating (X) is given by the expression:

$$\log. Y = 20{\cdot}0468 - 0{\cdot}1289 X.$$

Partial freezing does not affect creaming ability, but the behaviour depends on the manner of thawing (128).

Decreasing the dispersion (or hydration) of the casein of cream by adjustment of the *p*H, *e.g.* towards its isoelectric point (by adding mineral acid), lessens the time of churning (129). A study of the influence of the substances adsorbed on the fat globules on churning, by determining the churnability of artificial emulsions with the known emulsifying agents of cream has been carried out by Wiese and Palmer (130). The artificial emulsions were prepared with the following emulsifying agents: calcium caseinate-phosphate, globulin-lecithin, lactalbumin-lactose-globulin-lecithin and buttermilk. The emulsions were prepared by homogenising, separating and standardising the cream to 30–35 per cent. fat. On churning, only the buttermilk emulsion gave normal butter and thus none of the above agents is the adsorbed material on the fat globules. Fresh buttermilk therefore contains the fat stabiliser which is lost from the fat globules when the emulsion character is changed during churning. The stable emulsions showed different behaviours during separating and churning. Thus, lecithin gave the best separation and lactalbumin-lactose the best churning while the casein emulsion was only partly churnable. Cream which yielded a small amount of foam, and cream freed from milk plasma by washing, churned easily.

Krenn (131) gives, as the average electrical conductivity of milk, 42×10^{-4} mhos with the lowest value $35{\cdot}1 \times 10^{-4}$ and the highest value $105{\cdot}1 \times 10^{-4}$. Plucker (132) gives a range of *normal* milk from $39{\cdot}3 \times 10^{-4}$ to $56{\cdot}3 \times 10^{-4}$. The usual range quoted previously was from $37{\cdot}7 \times 10^{-4}$ to $41{\cdot}7 \times 10^{-4}$. Roeder (133) has examined the effect of neutralisation, or of added alkali, carbonate or bicarbonate, on the conductivity and provides tables of results.

The Wurster range of the freezing point depression in milk (0·537–0·560°) is exceeded by from 0·005° to 0·007° by some cows, in oestrus (132). A new index of milk quality (depression of the freezing point + (0·00184 × millimols of NaCl per litre)), which, for normal milk ranges from 11 to 15, has been suggested (134). Saito (135) reports the freezing point depression in milk from Japanese cows as: Holstein 0·555°, native breed 0·551°. The freezing point depression is roughly proportional to the increase in acidity, but lactic acid is not the only factor. Golding (136) quotes a range of 0·544–0·564° for 91 samples of milk varying in content of solids-not-fat from 6·60 to 9·05 per cent. (Hortvet method). Elsdon and Stubbs (137) have contributed a review of the findings of various investigators on the determination of the freezing point of milk, and on its uses as a means of detecting added water—for which it is an extremely valuable test.

Mohr and Brockmann (138) have studied the cataphoretic behaviour of cow's milk and find that a relationship exists between the butterfat content and the charge on the particles. The optimum migration of fat occurs in media where the *p*H is close to the isoelectric point of the fat particle. In pure aqueous emulsion the isoelectric point of the fat particle is on the more acid side than in milk owing to the presence of casein in the latter.

Light transmission through thin films of milk is proportional to the concentration of casein; the fat phase has little effect but individual variation is experienced (139).

(*b*) Instability to heat.

Holm *et al.* (140) have studied the heat coagulation of milk in relationship to its chemical composition over a lactation period. No high degree of correlation could be found between heat stability and total solids, fat, ash, Ca, Mg, P, citric acid, excess base content, titratable acidity, *p*H, buffer capacity or stability to alcohol and phosphate. It appeared that the heat stability of fresh and evaporated milks (up to 18 per cent. total solids) increased as the Ca + Mg content rose, this change occurring as lactation advanced. A variation in the buffer capacity of each individual milk was noted. Further work on the influence of various salts (141) included an attempt to stabilise (or destabilise) colloidal dispersions of calcium caseinate with electrolytes. The solutions failed to respond in a regular manner. The times of coagulation of skim milk (9 and 18 per cent. total solids) at temperatures from 80 to 130° C. showed that they decreased logarithmically with increase of temperature. Forewarming, the addition of aluminium chloride, and of small amounts of acid was found either to increase or to decrease the stability. In studying the effects of added salts, it was found that milks could be divided into two classes, one being stabilised by the addition of positive ions, the other by negative ions (*e.g.* citrate or phosphate neutralisers). The heat stability of a concentrated milk could not be judged from that of the original as, with samples of lower concentrations of solids-not-fat, the various electrolytes in the samples exhibited considerable differences in effect throughout their ranges of concentration.

The phosphate test for milk unstable to heat (142), which, as mentioned above, is not applicable in all cases, depends on the combined action of added acid, salt and heat. Data have been obtained showing the relationship between heat stability and the minimum concentration of phosphate necessary to produce initial coagula-

tion in milk and when the coagulation shows low heat resistance. A phosphate number is suggested. By excluding milks with low numbers greater heat stability was obtained in the bulked milk. Remarkable constancy in herds was observed. There was no relation between *p*H and the phosphate number. Homogenisation of cream lowers its stability to heat (143), but a second homogenisation at lower pressure (500 lb.) increases the stability.

Whitaker (144) attributes the feathering of evaporated milk in coffee to an incorrect salt balance, to a highly concentrated coffee, to prolonged extraction of coffee grounds, to the use of small quantities of milk and/or to the slow rather than the quick addition of the milk. Doan (145) observes that acidity and homogenisation favour the feathering of cream in coffee and advises the use of pasteurised sweet cream, homogenised at 1000 lb. with a lower pressure at the second stage. Stabilisers such as citrate, phosphate or carbonate (0·025–0·10 per cent.) may be added. The mechanical destruction of fat clumps by homogenisation increases the heat stability of cream, the fat clumps being believed to act as nuclei for the separation of casein. Cream should not be homogenised below forewarming temperatures unless preheating has been done at 170° F. for 30 minutes or 180° F. flash.

(*c*) Rennin coagulation.

Porcher (146) has investigated the effect of acidity, calcium and phosphate concentration, heat, and carbon dioxide on rennin coagulation and curd tension. Heating and decalcifying salts lower curd tension or even prevent coagulation. Bubbling carbon dioxide into casein solutions causes a soft acid curd but a firm rennin curd. For a constant amount of rennin, different amounts of casein require proportionate amounts of calcium for coagulation. Calcium caseinate in solution with paracaseinate prevents the rennin from coagulating paracasein. Blood serum can control the curd tension of rennin coagula from reconstituted or heat-treated milk. Albumin (in colostrum and human milk) lowers curd tension.

Moir (147), investigating the effect of heat on rennin coagulation, suggests that the decreased coagulability of heated milk is partly due to flocculation of soluble milk protein on the casein micelles.

Christen (148) has observed that the law of rennin action (concentration of rennin multiplied by time of coagulation = constant) does not hold when tested on a calcium caseinate-phosphate complex. With such artificial solutions the "constant" increases as the rennin concentration decreases, while with milk it decreases with decrease of rennin concentration. Grimmer (149) has found that dilution of rennet does not decrease the rate of enzyme action if the dilution is allowed for. Schneck (150) finds that the completeness of clotting of cream by rennet is represented by the expression $(100-F)F_1m$ per cent. (F = per cent. of fat in whole milk, F_1 = that in the skim milk and m = per cent. of skim milk). Heating above 37° C. causes an increase, but below 37° a decrease, in the degree and rate of clotting. Homogenisation of cream decreases its stability to rennet (143). Beau (80) suggests that rennin coagulation consists of a polymerisation or a physico-chemical condensation of casein and calcium phosphocaseinate.

(*d*) Casein salts.

Calcium caseinate is dispersed in skim milk in sizes ranging from molecular (4 mμ) to 200 mμ. Heating to 95° C. has no effect on the dispersion (151). The *p*H of milk is altered by boiling with calcium chloride, casein being precipitated in the *p*H range 5·95–6·05. Precipitation occurs at the point of maximum adsorption of calcium (152). The addition of the amount adsorbed to boiled milk causes rennin coagulation to proceed normally. The cations Ca > Ba > Mg stimulate coagulation of raw and boiled milk in this order, whilst K and Na have no effect. The binding of alkali to casein

and paracasein in excess of that of stoichiometric combination is due to adsorption, and the effect of adding neutral monovalent salts is to displace the *p*H to the acid side, this being more apparent at *p*H 6–9 than at *p*H 11–12·5 (153).

The acidity of milk to which oxalate has been added depends largely on the colloidal (mainly tricalcium) phosphate content (154, 155).

(*e*) Acidity and acid-base equilibria.

The effect of dilution on the titratable acidity of milk has been investigated by Sommer and Minos (156), who find that the acidity is lowered by 0·0211 per cent. with an equal volume and by 0·0623 per cent. with 9 volumes of water. In solutions containing calcium and phosphorus in the amounts found in milk the precipitation of $Ca_3(PO_4)_2$ will occur, giving a larger titration value so that the effect of dilution of milk is to decrease the amount of precipitation of phosphate. A relationship of calcium to caseinate similar to that of calcium to phosphate is involved in the dilution effect. A lower *p*H is observed at the phenolphthalein endpoint with diluted milk due to the protein-effect on the indicator and to the rapid fading which results from precipitation of $Ca_3(PO_4)_2$. The acidity of concentrated milk is higher than the value calculated from the acidity of the original milk and the amount of concentration in spite of loss of carbon dioxide is due to a reversal of the above process. The neutralisation of milk or cream with lime does not lower the acidity to the calculated point because the higher Ca concentration causes greater precipitation of $Ca_3(PO_4)_2$.

Grimmer *et al.* (157) find that in the progressive souring of milk the calcium content of the coagulum diminishes regularly whereas with rennin coagulation Ca and P are co-opted or a combination of $Ca_3(PO_4)_2$ with casein occurs. The hydrogen ion concentration of milk (Y) is connected with lactic acid concentration (X per cent.) by the formula $Y = KX^2C$, where C is the hydrogen ion concentration of lactic acid of acidity X and K is a constant. K was found equal to 3·03–5·42 (average, 4·3) $\times 10^{-6}$.

The addition of formaldehyde to milk increases the buffer capacity. More acetic acid is therefore required to reach the isoelectric point, and a greater concentration of trichloracetic acid is necessary for total protein precipitation (158).

Watson (159) suggests that the higher buffer value of Jersey milk over that of Holstein milk is probably due to the higher solids-not-fat content of the former. Pasture has no effect on the buffer value. Nakayama (160) ascribes the buffering action of milk to its casein content and to changes due to protein decomposition.

(*f*) Oxidation-reduction potential.

Buruiana (161) finds the E_h of fresh milk to be $+0{\cdot}200$ volt, which, by the addition of aldehyde or by the action of bacteria is lowered to $-0{\cdot}200$ volt. Heating is also found to reduce E_h and lactose is suggested to be responsible.

Aikins and Fay (162) find that the time-potential curves with and without methylene blue are in close agreement during the entire reducing process and that the blue colour can be restored on shaking with air. This confirms Whitehead's findings (26). The flora of market milk influences the type of curve. With an increase in the percentage of fat the zone of reduction becomes more positive, but it becomes more negative with an increase in the concentration of the dye and the time taken for bleaching is delayed. The potentials of cream, milk and skim milk drift to the negative side when exposed to sunlight, methylene blue accentuating the change; the addition of fat, since it heightens the zone of reduction, also hastens the change. The reduction intensity due to bacteria is more negative than that due to light, and the reactions are therefore different. When skim milk containing methylene blue is

bleached by sunlight and then placed in the dark, the potential becomes sufficiently positive to oxidise the bleached dye.

Martini (163) states that milks from different species show different powers of reducing methylene blue in light, the most active being sheep's milk, while cow's milk loses its power readily. The power is not associated with the fat but with a thermostable water-soluble constituent—probably glutathione—which is present in sheep's milk in twice the amount that is found in cow's milk.

IV. MILK PRODUCTS.

(*a*) Butter.

Davel (164), reporting on the manufacture of butter under South African conditions, states that cream for churning must be of less than 0·5 per cent. acidity but that butter from pasteurised cream of 0·4 per cent. and less keeps better. Lime gives the best result as a neutraliser, the neutralisation being carried down to 0·3 per cent. only, whilst bicarbonate can be used to neutralise down to 0·25 per cent.

Lucas *et al.* (165) have found that acidity, or the amount of added starter, has no effect on the curd content of butter and that the method of churning has little effect. An increase in the acidity of the starter increases slightly the acid value of the fat. Ripened cream butter deteriorates more rapidly during storage, that from cream of 0·45 per cent. acidity deteriorating first and developing an unclean fishy or tallowy taste.

Lampitt and Sylvester (166) have found that air and light increase the susceptibility of butter to autoxidation and its attendant taints. Wright and Overman (167) state that the period of induction is shortened by various factors in the previous treatment of cream, *e.g.* copper contamination, acidity and high temperature. Oxidation is influenced by the same factors, the effect of copper being most pronounced, whilst acidity also has an effect, though not so marked as copper and high temperature.

Wiley (168), investigating the wood taint in butter boxed in hoop pine (*Pinus radiata*), has found that volatile terpenes from the wood diffuse into the butter to the extent of 1 to 10 p.p.m. and thus cause the taint. Waterproof cellophane, or spraying the inside of boxes with a formalin-sodium caseinate solution had a satisfactory protective action.

The aroma of butter is due to the diketone, $CH_3CO.CO.CH_3$ (diacetyl), which is an oxidation product of acetyl methyl carbinol. Quantities varying from 0·1 to 0·6 p.p.m. have been found in fresh butter. The carbinol is a metabolic product of some of the lactic acid bacteria (169). It is probable that the carbinol only exists in starter and cream owing to the low E_h values of such media. The milk fat dissolves the carbinol and, after making the butter, oxidative changes convert the carbinol into the ketone, which produces the aroma. Acidity of cream is essential for aroma production since butter from sweet cream does not contain any diacetyl. The oxidation of the carbinol during the storage of butter initiates oxidative changes in the butterfat and butters of strong aroma have low keeping quality. King (170) has found that diacetyl causes tallowiness and bleaching of butter exposed to air and light at a rate proportional to the amount present. It is probable that, in practice, the increased acid value of the fat of butter from ripened cream increases the tendency to autoxidation as well. Tapernoux (171) also notes the development of tallowy flavours in high-flavour butter. The origin of diacetyl in butter and its addition to butter and margarine as a flavouring agent have been described (172).

Ciusa (173) has observed the formation of diacetyl and the carbinol by the action of pepsin on milk. Digestion for 24 hours at 38° C. yielded 0·087 per cent. The nickel-glyoxime method has been used for the determination of diacetyl (174).

The changes which take place in butter on ageing occur mostly in its content of volatile fatty acids, decreases in the Reichert-Meissl and Polenske values being appreciable (175). Elsdon *et al.* (25), on the other hand, find that butterfat exposed to light and air for 19 weeks does not change appreciably in Reichert-Meissl value, but that margarine fat increases in Reichert-Meissl and Polenske values, so that the apparent butterfat content, if present, may well seem above the legal limit in amount.

The influence of working on the moisture content of butter has been investigated by Otte (176), who finds that, in unworked butter, the moisture content increases as the size of fat particles, with acidity and with the time of holding cream at low temperatures. Working gradually decreases, but over-working increases the moisture content. The temperature of wash water and the salt content influence the adhesion of water whilst summer butter shows the maximum adhesion.

The consistency of butter varies partly with the iodine value of the fat and partly with the methods of manufacture. Low iodine values give a soft butter, but long cooling of the cream previous to churning and cooling of the granules before working may overcome the differences due to iodine value (177).

Hunziker *et al.* (178) determined the *p*H of butter on the curd serum. Nissen (179) states that the *p*H of the curd serum gives a truer index of the reactions occurring in churning. No agreement between *p*H and acidity of butter has been observed.

King (180) has investigated the relationship between the structure of butter and the chemical changes in the fat. The continuous phase is a liquid fat in contact with water droplets, and chemical and bacteriological changes occur at the fat-water interface. The effective area of contact with water can be calculated from the diameters of the water droplets. The water droplets are composed of the buttermilk. They have a greater boundary surface than the wash water and therefore cause greater spoilage. The metals dissolved from poor equipment are responsible for more spoilage than the wash water.

(*b*) Cheese and whey.

Wode (181) has studied the relation between calcium content and the *body* of cheese and finds that high calcium content is accompanied by a weak body and *vice versa*. The quantity of calcium dissolved from the curd increases with the acidity of the milk before setting, and the quick separation of vat whey means less loss of calcium. The total calcium dissolved by vat whey is greater than that by press whey. A higher acidity in the milk also shows less calcium in cheese ash (182). Moir (183) has also observed that the depletion of calcium and phosphorus from the curd is greater by the more acid whey from pasteurised than from raw milk.

The *p*H of cheese has been determined on the cheese itself and on the juice by using the quinhydrone electrode. The centre of Tilsiter cheese is the most acid (*p*H 5·2–5·6), the outer zone decreasing during ripening until neutral (184). Davis (185) finds that the oxidation-reduction potential is not uniform throughout the cheese but is more to the oxidative side around cracks, borings and the rind. The distribution of moisture and salt has been found to vary in parts of the same cheese and in cheese from the same vat. Salt diffuses very slowly in hard cheese (186).

Sadler and Eagles (187), investigating the ripening of Kingston cheese, find considerable increases in water-soluble nitrogen, non-protein nitrogen, amide and amino-nitrogen. After the first few hours, the protein breakdown is of a peptic-tryptic nature. Moir (188) finds that the amount of decomposition products are lower in Cheddar cheese from clean or pasteurised market milk. The blueing of Stilton cheese is favoured by a low content of volatile acids in the press juice (189).

The gases of cheese contain only traces of hydrogen, and consist mostly of carbon dioxide (96 per cent.) and nitrogen. The gas obtained by evacuation of the curd is 99·9 per cent. carbon dioxide (190).

The following average moisture contents of Continental cheese have been reported: Emmenthal 35, Dutch 43, Tilsiter 43, Limburger 50, Camembert 55 per cent. (191).

Cases of muddy discoloration, black spots due to particles of lead or solder, and bleaching of coloured cheese have been reported.

The blackening of the inside of tinfoil wrappers of processed cheese (at *p*H 5·8) is due to the solution of the tin and its precipitation as stannous oxide by anodic oxidation (192). The hardness of processed cheese is controlled by the water content, which should be from 40 to 43 per cent. Higher water contents give a soft cheese. The water content of the original cheese can be increased from 6 to 9 per cent. in the melting process. The solutions for the melting process are either carbonate or a carbonate-citric acid mixture.

(*c*) Dried products.

Marquardt (193) states that the solubility of skim milk powder decreases slightly on exposure to light and air and that on storage, which is best done in sealed containers, a slight change of the *p*H to the acid side occurs. The insoluble portion in high-soluble powders is protein but may be protein *plus* lactose in low-soluble samples. The texture of ice-cream from low-soluble powder is improved by high pasteurisation temperatures but age and solubility have no effect on the quality of either ice-cream or cream cheese (194).

Wright (195) observes that the solubility of roller-dried milk varies with the temperature of reconstitution, showing relatively high solubility at high temperatures. The heating of milk of high solids content tends to make the protein insoluble, this being more rapid as the solids in the milk increase. Thus, half the protein was rendered insoluble by heating at 100° C. for 33 minutes when the solids content was 28 per cent.; for 48 per cent. solids only 2 minutes were required and for 88 per cent. solids only 0·2 second. Skim milk powders behave in a similar manner.

In determining the solubility, the temperature of reconstitution is important, since extreme differences in solubility may be met with at various temperatures. Some protein is rendered reversibly insoluble by heating in the dry state and temperatures up to 50° C. will cause rehydration and solution; the rest of the protein has been irreversibly coagulated by moist heat. Heat treatment during reconstitution may render protein insoluble at temperatures above 60° C., so that, for the determination of solubility, temperatures above this should not be used. The fat content of the powders does not materially interfere with the results (196).

Lampitt and Bushill (197) have observed that organic solvents extract almost all the fat of roller-dried milk but only from 3 to 14 per cent. of that of spray-dried milk, which is increased to 83 per cent. by grinding. The behaviour of spray-dried powder when absorbing moisture is remarkable; it first becomes clammy and then hard and powdery, at which stage the fat enters the "free" condition due undoubtedly to the crystallisation of an amorphous film of lactose which surrounds the fat, and protects it from solution by organic solvents. The "critical moisture" range is proportional to the solids-not-fat content of the powder. The presence of amorphous lactose in dried milk products is thus an advantage, since free moisture will be gradually used up to hydrate the amorphous form.

(*d*) Ice-cream.

The processing of ice-cream mix at 170–180° F. causes a decrease in the degree of fat clumping and viscosity and an increase in protein stability. Homogenising should be carried out at 150° as this vitiates some of the effects of processing at 180°. A high processing temperature has more effect on viscosity, overrun and protein stability than a high homogenising temperature, which has the greatest effect on fat clumping (198).

V. PROCESSING.

(*a*) Metallic corrosion and flavours.

The work on the general chemistry of the processing of milk products has been dealt with in the preceding sections and the pasteurisation of milk has been fully dealt with in a previous review (199).

Metallic contamination during processing has been the subject of much investigation. Popp (200) reports the following increasing order of resistance of metals to corrosion: Ni, Cu, Fe, Sn, Al, a most resistant alloy being that of Fe, Ni and Cu. Gebhardt and Sommer (201) state that a slight increase in acidity has no effect, while high acidity (pH 3·3) markedly decreases the solubility of copper. The solubility of copper increases to a maximum at 70° C. irrespective of the length of time of exposure, and at the boiling point of milk the rate of solution is the same as at room temperature. The oxidation-reduction potential of the milk is related to the solubility rate of copper. Up to 60–65° the oxidation-reduction potential remains constant, but it decreases rapidly above 70°.

The measurement of residual currents given by electrodes of various metals in milk has been used as a method for the qualitative measurement of the rate of solution of those metals in milk (202). The protective film on metals and alloys (Ni, Cu, Cr-Ni alloys and nickel silver) prevents much corrosion while heating milk, but when hot milk is cooled corrosion can occur owing to the absence of the film. The protective film depends on the oxygen content, velocity of flow and the temperature of the milk. Copper surfaces are not noticeably protected during heating in the same way as nickel and nickel alloys. The effect of galvanic corrosion is unimportant (203). Fink and Rohrman (204) show that copper, nickel and copper-nickel alloys may bring about either an increase of 0·7 p.p.m. or a decrease below 0·3 p.p.m. in the copper content of milk. The replacing effect of Ni on Cu is more noticeable at high temperatures. Chromium-nickel steels, aluminium-nickel alloys, chromium-plated copper and chromium-plated nickel are very resistant to corrosion by milk.

Guthrie *et al.* (205) have confirmed the opinions of previous workers that traces of copper entering milk from copper or copper alloy surfaces cause an oxidised flavour. Chromium-plating is inferior to tin-plating in resisting corrosion. Davies (206), investigating the metallic contamination of milk during processing, finds that in the majority of cases the cooler is responsible for the greatest addition of copper. A minimum of 1·5 p.p.m. of copper will cause an oxidised flavour to develop within 24 hours in milk of good hygienic quality stored at low temperature. Iron contamination of milk during processing runs parallel to copper contamination.

Osterburg and Liepus (207) have published a review of the literature on metals in the dairy industry (200 references).

Various flavours in market milk have been described. An oxidised flavour termed "emery taint" (28) and a rancid flavour due to homogenisation (208) have been reported. The occurrence of traces of trimethylamine in the milk of cows fed on sugar-beet tops causes a fishy or "beety" flavour to be detectable in milk (209).

Carbonation of milk gives variable results on keeping quality but carbonation at high pressure gives an increased keeping quality (210).

(*b*) Pasteurisation.

Patents have been taken out for the sterilisation of milk with a low tension direct current (211). Although the main effect is due to the heat generated, Gelpi (212) maintains that a factor other than heat is operative, since no spores would germinate after the "electropure" process.

The pasteurisation of milk may be evaluated according to the following criteria: efficiency of bacterial destruction, imparting of cooked flavour, degree of albumin coagulation, increase in rate of acidity development in the first 3–5 hours' incubation, maintenance of the original acidity of the milk and the carbon dioxide content, the retention of the original ability to coagulate with rennet, the extent to which creaming ability is affected and the effect on the vitamins and ash constituents. Pasteurisation in bottle, a process which has recently been developed (213), embraces less pumping and agitation of the milk with its attendant difficulties (foaming and loss of creaming ability), no loss of carbon dioxide and no incorporation of air, more effective destruction of bacteria and the absence of metallic flavour.

The stassanising process involves the passage of milk between two pipes, one inside the other, with hot water at 76·5° C. passing through the inner and outside the outer pipe. The natural carbon dioxide content of the milk is preserved, the calcium salts are not precipitated and a 99·4 to 99·9 per cent. destruction of bacteria is claimed.

Various tests for pasteurised or insufficiently pasteurised milk have been devised. The adsorptive action of the fat globules on colloidal particles such as clay, carmine, red blood corpuscles etc., is lost on heating above 58° C. The Schern-Gorli test takes advantage of this property (214) using, preferably, charcoal, indigo, or carmine. Since the peroxide test (Storch's reaction) may be applied to distinguish between low and high temperature pasteurisation, this test is claimed to be applicable for the detection of milk insufficiently pasteurised at low temperature. Milk of high acidity or skim milk does not give the test.

The detection of insufficiently pasteurised milk by the amylase test has already been described (91) (Section II (*d*)).

A standardised rennet test, observing the time of coagulation, has been suggested by Ladanj (215). Two ml. of rennet (1:100,000) in 50 ml. of milk at 35° C. will coagulate raw milk in 3 min., and pasteurised milk in 3–10 min. while boiled milk requires over 10 min.

Milk stone has been found to have the following composition: fat 3·6–17·7, protein 4·1–43·8, ash 42·0–67·3 per cent. of dry matter. Low temperature heating to avoid local coagulation of milk proteins and the use of washing water which does not give a precipitate with alkaline detergents is advisable (216).

The creaming of heated milk has been dealt with in Section III (*a*). The rate at which the milk is cooled is of major importance. Slow cooling (2–4 hours) seriously decreases the volume of cream layer but this is less noticeable at 24 hours. One hour's cooling, as is usually employed, does not seriously reduce the cream line.

VI. ANALYSIS OF MILK AND ITS PRODUCTS.

(*a*) Fat.

Non-centrifugal methods, embodying the principles of the Hoyberg method, have been advocated for the determination of the fat in milk, ice-cream and condensed milk. Singh (217) suggests the separation of the fat from milk with an alkaline-tartrate-methyl-butyl alcohol mixture in a Babcock bottle standing in hot water, the fat being read off on the neck. Petersen (218) has patented a modified Hoyberg method. Overman and Garrett suggest a non-acid Babcock method for determining fat in ice-cream. They use two liquids: (A) 75 ml. C.P. NH_4OH, 35 ml. butyl alcohol, 15 ml. 95 per cent. ethyl alcohol; (B) 200 g. Na_3PO_4, 150 g. CH_3COONa in 1 l. of water. Nine g. of ice-cream are used in a 8–10 per cent. Babcock bottle and the test is carried out as in the Hoyberg method. The same method has been suggested for the fat determination in condensed and evaporated milk (219).

For more accurate determinations, the Schmidt-Bodzynski method has been advocated. This consists of boiling 10 ml. of milk with 10 ml. of hydrochloric acid, previous to a mixed ether extraction such as is employed in the Roese-Gottlieb process (220, 221).

(*b*) Total solids.

The calculation of the total solids from the specific gravity and fat content by the Richmond scale has been found accurate for summer milk but may be 0·4 per cent. too high for winter milk. Fleischmann's formula gives less accurate results. The specific gravity of milk increases for the first 12 hours but the change is influenced by the temperature at which the milk is kept (222).

Evaporation, *in vacuo* over sulphuric acid in desiccators, of milk spread over asbestos fibre in small weighing bottles has been recommended by Hawley (223). The method is useful for humid atmospheres and high night temperature while keeping the evacuated desiccators in an incubator overcomes the difficulty in temperate climates.

(*c*) Preservatives.

The Hehner test for formaldehyde has been applied either as a zone reaction or as a uniform colour throughout the liquid. For the zone reaction, 1 ml. of the milk is carefully pipetted on to the top of a 3:1 sulphuric acid solution containing potassium bromide; a violet zone quickly develops if formaldehyde is present. For the general reaction dilute sulphuric acid (8 to 5 of water) is mixed with a quarter of its volume of milk, cooled, and 0·5 ml. of a mixture of equal parts of concentrated sulphuric acid and bromine water added. A violet colour forms at once if formaldehyde is present (224).

(*d*) Added water.

Cerf (225) has reviewed the commonly used methods for detecting added water in milk. He states that the refractivity of milk serum, freezing point, lactose *plus* 5 × ash, and the specific molecular constant are the most satisfactory methods. The nitrate test (Lerrigo) for added water depends on the detection of very small amounts of nitrates which generally occur in natural waters. As Lerrigo's test, involving the use of diphenylamine, requires a certain amount of nitrate to oxidise the amine to the intermediate for dye formation (diphenyl-benzidine), Monier-Williams (226) advocates the use of the latter compound in the test. This reagent shows a gradation of colour for from 0·1 to 1·0 p.p.m. of nitrate. Drenching cows with potassium nitrate does not cause any nitrate to appear in the milk but the practice of adding a small quantity of nitrate to milk to neutralise turnip-flavour naturally invalidates the test (227).

Elsdon (228) states that refractometric measurements are best done on the copper serum, but if whey from soured milk is used, the souring must be carried out at uniform temperature and the reading taken as soon as complete clotting occurs. Beckel (229) states that the addition of 15 ml. of 17·5 per cent. copper sulphate solution to 30 ml. of milk gives a serum which shows the addition of added water more sensitively than the calcium chloride serum.

The ash of normal milk contains 2·0 per cent. carbon dioxide. The addition of bicarbonate to milk can be detected and approximately determined from the excess of carbon dioxide in the ash over this value (230).

(*e*) Chlorides.

A variety of open Carius methods have been suggested for the determination of chlorides in milk and dairy products. The material is digested with nitric acid in the presence of excess silver nitrate until all protein is disintegrated. The excess silver nitrate is then determined by Volhard's method (231, 232).

(*f*) Fat in buttermilk.

Bird and Sands (233) found that from 15 to 26 per cent. of the fat determined by a Roese-Gottlieb method on buttermilk was lipin (0·107–0·176 per cent.). The drying of the buttermilk causes some lipin destruction. Sulphonation of the fat in the Babcock method causes a greater error than that due to lipins. The lipin concentration in 20 to 40 per cent. cream is not greater than the usual seasonal variation in fat content. The fatty acids of the fat separated by the Babcock test are more unsaturated than those of the fat of normal milk. Lyons and Finlay (234) find that the Roese-Gottlieb method gives higher results than the Gerber method for fat in buttermilk. Added lecithin increases the Roese-Gottlieb and the Gerber values.

(*g*) The determination of milk proteins.

Moir (50) has applied methods commonly used for protein separation to the accurate determination of the protein distribution in milk. *Total nitrogen* is determined on 5 g. of milk. *Total protein* is precipitated from a final concentration of 4 per cent. trichloracetic acid at 60–65° C. for 30 min., with subsequent cooling for 30 min. Five g. of milk are used. *Casein* is precipitated from about 10 g. of milk by the addition of acetic acid-sodium acetate buffer to *p*H 4·75 in the following manner: The diluted milk (50 ml.) is first made acid with 1·5 ml. of 10 per cent. acetic acid. The mixture is allowed to stand for 20 min. and 4·5 ml. of 0·25 *N* sodium acetate are then added. Filtration is carried out after standing for 1 hr. The precipitates are washed with distilled water. Casein *plus* globulin are precipitated with saturated magnesium or sodium sulphate solution from 10 g. of milk, an extra amount of the powdered salt being added to saturate the water of the milk. Washing of the precipitate with the saturated precipitant is effected by flooding the precipitate on the filter. All precipitates are digested by the Kjeldahl method and the nitrogen distilled in the usual way. The four unknowns: casein, albumin, globulin and non-protein nitrogen can be determined from the above four determinations.

(*h*) Cryoscopy.

Rothenfusser (235), Weinstein (236), and Elsdon and Stubbs (237) have described the technique of the test with various improvements. Elsdon recommends the Hortvet apparatus and discusses possible sources of error in the determination. It is impossible to make an exact correction for increases in acidity, but an approximate estimate of the true depression can be obtained by subtracting 0·003 from the observed value for each 0·1 ml. over 2·0 ml. of 0·1 *N* soda used for 10 ml. of milk. The degree of supercooling is important. This should be in the neighbourhood of 1° below the probable freezing point. A higher degree of supercooling requires a significant Raoult correction (0·018 for 2°). This is of the same order of magnitude as the interpretable differences between the freezing points of genuine and, say, 10 per cent. watered milks.

A tendency for the freezing point to fall slightly when milk is heated has been experienced, but the change is so small as not to interfere with the interpretation of the results. One sample of milk has been heated to 230° F. in an autoclave without change of the freezing point being detectable (238).

(*i*) Miscellaneous.

Calcium saccharate (viscogen), used as a cream thickener, combines with phosphoric acid to form colloidal calcium phosphate. The addition of soluble oxalate changes the gelatinous phosphate to crystalline calcium oxalate, thus giving a cream

of greater fluidity. To detect viscogen, Pyne (239) determines the viscosity before and after the addition of sodium oxalate solution. Some cream samples do not thicken much with viscogen and will therefore not give very positive results.

The use of ultra-violet light for detecting fats and other material by the fluorescence obtained has been applied to the examination of dairy products. Stark (240) has found that milk protein under the light of a quartz-mercury lamp is white to yellow with a bluish cast, pus is dark yellow, curd of unripe cheese is a mat yellow, and ripe cheese is blue-grey with a violet tone. Molds are fluorescent. Milk fat is a strong yellow and white, but vegetable fat is violet to blue with fluorescence. Braunsdorf (241) observes that milk and homogenised cream give a yellow fluorescence changing to a white with a bluish tinge on ageing. Dried whole milk powder, cream powder and skim milk powder are white with a blue fluorescence, and emulsions of vegetable oils give the same fluorescence as the oils alone.

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